# *Abstracts*
# *The American Society for Cell Biology*
# *45th Annual Meeting*

**December 10-14, 2005**
**The Moscone Center**
**San Francisco**

# CONTENTS

## SATURDAY



## SUNDAY



## MONDAY



## TUESDAY

# CONTENTS



## WEDNESDAY

## Big Science, Little Science (1-2)

1

### Unraveling Smell

L. Buck; Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, WA

Odorants are detected by ~1000 different odorant receptors (ORs), which are located on olfactory sensory neurons in the nose. Our studies show that ORs are used combinatorially to detect different odorants and encode their unique identities. To explore how the nervous system translates odorous chemicals into perceptions, we asked how inputs derived from different mouse ORs are organized as signals travel from the nose to the olfactory bulb and then the olfactory cortex. In the nose, each sensory neuron expresses a single OR gene. Neurons with the same OR are dispersed in the nose, but their axons converge in a few glomeruli at two fixed locations in the bulb. The result is a stereotyped sensory map in which inputs from different ORs are segregated in different glomeruli and relay neurons. In the olfactory cortex, inputs from one OR are targeted to clusters of neurons at specific sites, creating a stereotyped map unrelated to that in the bulb. In contrast to the segregation of different OR inputs seen in both the nose and bulb, it appears that different OR inputs overlap extensively in the cortex and single neurons receive combinatorial inputs from multiple different ORs. Using c-Fos as an indicator of neuronal activator, we found that different odorants elicit different, but partially overlapping, activation patterns in the cortex. The representation of each odorant is composed of a small subset of sparsely distributed neurons. Quantitative analysis of the odor representations suggests that cortical neurons may function as coincidence detectors that are activated only by correlated inputs from different ORs.

2

### Discovery-Driven Research: A New Frontier in the Biological Sciences

C. Fraser; The Institute for Genomic Research, Rockville, MD

The application of large-scale approaches to the study of biological questions has produced a fundamental change in the way that we approach scientific discovery. In this new era, high-throughput technologies are providing enormous amounts of new data and computational biology is allowing us to make links between genome sequence and biological processes and function. The ultimate goal of such big science is to achieve a predictive understanding of biology.

## Quantitative Studies of Cell Signaling Networks (3-4)

3

### The Deeper Correlations: Single Cell Measures of Kinase Signaling for Mechanistic and Clinical Analyses

G. Nolan; Microbiology/Immunology, Stanford University, Stanford, CA

Intracellular assays of signaling systems has been limited by an inability to correlate functional subsets of cells in complex populations based on active kinase states or other nodal signaling junctions. Such correlations could be important to distinguish changes in signaling status that arise in rare cell subsets during functional activation or in disease manifestation. Simultaneous detection of activated kinases and phosphoproteins in simultaneous pathways in subpopulations of complex cell populations by multi-parameter flow cytometric analysis allows identification of signaling cascades for disease states by ordering of kinase activation and phosphoprotein status in signaling hierarchies. Importantly, we demonstrate that ordering of these activations requires multiple interrogations of cells, and that the networks discovered are reflective of deeper correlations. Using Bayesian Network analysis (a form of machine learning) one can infer pathway connectivity in an automated fashion, allowing for high throughput derivations of signaling system networks graphs in PRIMARY CELLS. The approach has powerful applications in mechanistic understanding, drug screening, and patient stratification for prediction of disease outcome in cancer, autoimmunity, infection, based on signaling network status. (1) Irish J.M., Hovland R., Krutzik P.O., Perez O.D., Bruserud O., Gjertsen B.T., **Nolan G.P.** (2004) Single Cell Profiling of Potentiated Phospho-Protein Networks in Cancer Cells. ***Cell***. 118:217-228. (2) Sachs K., Perez O., Pe'er D., Lauffenburger D.A and **Nolan G.P.** 2005. Causal protein-signaling networks derived from multiparameter single-cell data. ***Science***. 308:523-9.

4

### Systems Biology of Cytokine Signaling in Human Cells

P. Sorger, D. Lauffenburger, K. Janes, S. Gaudet, J. Albeck, B. Schoeberl; Department of Biology and Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA

Cytokines and their receptors activate complex signaling cascades to regulate cell proliferation, death and differentiation. We seek to develop quantitative, mechanistic models that describe cytokine-induced signaling with an eye to understanding cell-type variation and the differences between healthy and diseased states. We focus on decisions controlled by pro-apoptotic cytokines such as Tumor Necrosis Factor (TNF) and TRAIL and pro-survival cytokines such as EGF and the insulin-like growth factors (IGF). By mining a compendium comprising ~10,000 measurements of signal protein activities elicited by cytokines individually and in combination we can construct both statistical and physicochemical models. Classifier-based regression has been particularly helpful in establishing that cells respond to TNF directly, via activated TNF receptor, and indirectly via autocrine circuits involving transforming growth factor alpha (TGF- α), interleukin-1α (IL-1α) and IL-1 receptor antagonist (IL1-ra). These cytokines participate in a three-part autocrine loop that plays out over at least 24 hr and adds sequential layers of pro and anti-apoptotic signaling that sets cell death at a self-limiting level. Experimental work to date has been in human tumor cells. However, it seems highly likely that TNF-triggered autocrine cascades will differ from one cell type to the next. We are currently attempting to compare diseased and normal primary cells from breast and connective tissue. One interesting initial finding is that the logic of the TNF-TGF-IL-1α- IL-1ra cascade can be re-wired in some cell types by inflammatory cytokines such as interferons. There is much interest in the role of intracellular crosstalk among signaling circuits. We propose that that time-dependent crosstalk among synergistic and antagonistic autrocrine circuits may equally important. Morevoer, it should be easier to modulate the activity of autocrine than intracellular loops thanks to the increasing range of protein-based therapeutics available to target cytokines and their receptors.

## Bruce Alberts Award Presentation (5)

5

### Columbia University's Summer Research Program for Secondary School Science Teachers

S. C. Silverstein; Dept Physiology/Cell Biophys, Columbia Univ Coll Phys & Surg, New York, NY

Concerns about the quality of secondary science education stimulated me in 1990 to found Columbia's Summer Research Program (www.scienceteacherprogram.org). The program's purpose is to increase student interest and achievement in science by improving the quality of science instruction. To this end, Columbia's program provides secondary school science teachers with paid fellowships that support their participation in life and physical science research laboratories for two consecutive summers under the guidance of Columbia faculty. To date, 202 teachers have participated in the program. At ASCB's 2004 Education Forum, I reported that 8.4% more students in classes of participating teachers pass a NY State Regents exam in science than students studying the same subject in classes of non-participating teachers in the same school. This is objective evidence that teacher participation in Columbia's program has a significant positive impact on student achievement in science. The present value of this increase in Regents science exam pass rate is $11,782 per teacher in school costs saved annually, and $32,885 per teacher in additional tax revenues generated annually, yielding total annual economic benefits of each teacher's participation in Columbia's program that are 3.4-fold greater than the program's annual cost per teacher. **Policy implications of these findings:** A national investment of $75 million annually could support similar programs at 250 U.S. medical schools and research universities, while returning over $220 million annually in school costs saved and tax revenues generated. If each of these 250 programs enrolled 10 new science teachers annually, over 10 years they could provide science work experiences for 25,000 science teachers, approximately half the current membership of the National Science Teachers Association.

## Prokaryotic Origins of the Cytoskeleton (6-8)

6

### Bacterial tubulin homolog FtsZ

H. P. Erickson; Department of Cell Biology, Duke University Medical Center, Durham, NC

FtsZ is the major cytoskeletal protein in bacterial cytokinesis. When viewed by light microscopy it appears as a "Z ring" in the center of the cell. The Z ring constricts to divide the cell, disassembles during the constriction and then reassembles in the daughter cells. The substructure of the Z ring has not been visualized by EM, but we have turned to in vitro studies to deduce it. FtsZ assembles into short, single-stranded protofilaments (pfs), which are structural homologs of the tubulin protofilaments that make the microtubule wall. We have developed fluorescence techniques to study the kinetics of initial assembly and subunit turnover at steady state. FtsZ assembly is cooperative, showing a weak dimer nucleus and a critical concentration. (It is an enigma how a single-stranded pf can assemble cooperatively.) The assembly is very dynamic - pfs are turning over with a half time of 8 sec at steady state. The subunit turnover is regulated by GTP hydrolysis, and may involve a mechanism like microtubule dynamic instability. We believe that the Z ring in vivo is constructed from these dynamic pfs. This must involve a lateral association (the mechanism for this is unknown) and attachment to the membrane. FtsZ is tethered to the membrane by FtsA, a bacterial actin homolog. We used FRAP to determine the assembly dynamics of the Z ring in vivo. It is turning over with a half time of 8 sec, just as we determined in vitro. This is the most rapid cytoskeletal dynamics known. An important question is what generates the force of constriction? We have found in vitro that FtsZ can form curved pfs, which are equivalent to tubulin rings. The straight-to-curved pf conformational change is powered by GTP hydrolysis, and may generate the force for constriction.

7

**Dynamics of a DNA-Segregating Cytoskeletal System in Prokaryotes**

D. Mullins, C. Campbell, E. Garner; Cellular & Molecular Pharmacology, University of California, San Francisco, San Francisco, CA

The mechanisms that provide force to segregate bacterial chromosomes are still mysterious. We do, however, understand one important example of bacterial DNA segregation in molecular detail - segregation of the R1 and R100 drug-resistance plasmids. These large (100kb), low-copy plasmids encode genes for antibiotic and heavy-metal resistance and have been isolated from many pathogens. To ensure inheritance by both daughter cells during division, the R1 *par* operon constructs a simple DNA-segregating machine from three components. One of these components, ParM, is related to eukaryotic actins and assembly of ParM into actin-like filaments appears to drive plasmid segregation directly. We find that this simple prokaryotic cytoskeleton exhibits a remarkable collection of activities usually associated with eukaryotic cytoskeletons, including: dynamic instability, processive capping, insertional polymerization, and the ability to generate force. We also find that R1 plasmid segregation is a dynamic process in which assembly of unstable ParM filaments induces plasmids to oscillate rapidly from pole to pole of the cell producing a dynamic rather than static bipolar distribution. Our results indicate that the assembly dynamics of prokaryotic cytoskeletal systems are important to their cellular function and that the prokaryotic systems also make use of mechanisms similar to those of the eukaryotic cytoskeleton to establish long-range order and to move intracellular cargo.

8

**The Bacterial Cytoskeleton and Cell Shape**

C. Jacobs-Wagner,[1] N. Ausmees,[2] G. Charbon,[1] M. Cabeen[1]; [1]Molecular, Cellular & Developmental Biology, Yale University, New Haven, CT, [2]Uppsala University, Uppsala, Sweden

Similarly to eukaryotic cells, prokaryotic cells come in a variety of shapes. In eukaryotes, the cytoskeleton, which is made of microtubules, microfilaments and intermediate filaments, constitutes an internal framework that is essential for the maintenance of cell shape. For decades, it was thought that the external cell wall was the sole determinant of cell shape in bacteria. It is now apparent that bacteria also possess an actin-like cytoskeleton made of MreB and that this cytoskeleton is involved in determining rod cell morphology. Our laboratory has recently discovered a prokaryotic counterpart of intermediate filament (IF) proteins, termed crescentin. Crescentin is a fibrous protein with a tripartite domain architecture similar to that of metazoan IF proteins. Purified crescentin self-assembles spontaneously into ~10 nm wide filaments in vitro without exogenous energy sources, which is a distinct biochemical property of IF proteins. The function of crescentin is required for the characteristic crescent shape of *Caulobacter crescentus* as cells lacking crescentin lose their curvature and adopt a straight-rod cell morphology. Consistent with its role in cell curvature, crescentin forms a filamentous structure along the inner cell curvature of wild-type cells. Localization and proper organization of the crescentin cytoskeleton is dependent on the bacterial homolog of actin, MreB, indicating that MreB also plays an active role in cell curvature.

## E. E. Just Lecture (9)

9

**Still Waters Run Deep: Investigations into the Quiescent State in Yeast**

M. Werner-Washburne; Department of Biology, University of New Mexico, Albuquerque, NM

My laboratory has studied entrance into, survival during, and exit from stationary phase in yeast for the past 17 years. Previously, yeast and other microbes were thought to lack a true $G_0$ phase, because budded cells were always present in stationary-phase cultures. We have recently isolated two distinct cell populations from yeast stationary-phase cultures that contain very different cell types. We have concluded from our analysis that one fraction contains quiescent ($G_0$) cells and the other non-quiescent cells. The quiescent cells are generally the last cells formed after yeast cells exhaust glucose (the diauxic shift). They are refractile by phase contrast microscopy, unbudded, thermotolerant, and synchronous during exit from quiescence. Strangely, in the quiescent cells only nuclei and vacuoles are visible by EM. Non-quiescent cells contain many more membrane-bound organelles, including ER, Golgi, and mitochondria and lipid bodies and lack glycogen. Both cell types are metabolically active, but non-quiescent cells show a reduced ability to form colonies. We are continuing to characterize these cell types and their formation. The identification and characterization of these cell types provides the basis for ongoing, novel analyses of the quiescent state, asymmetric cell division, and the processes of cell differentiation and aging in yeast.

## Cargo Sorting & Vesicular Transport (10-15)

10

### An Intramolecular t-SNARE Complex Functions *in vivo* without the Syntaxin N-terminal Regulatory Domain

J. S. Van Komen, X. Bai, B. L. Scott, J. A. McNew; Biochemistry and Cell Biology, Rice University, Houston, TX

Membrane fusion in the secretory pathway is mediated by SNAREs (located on the vesicle membrane (v-SNARE) and the target membrane (t-SNARE). In all cases examined, t-SNARE function is provided as a three-helix bundle complex containing three ~70 amino acid 'SNARE-motifs'. One SNARE motif is provided by a syntaxin family member (the t-SNARE heavy chain) and the other two helices are contributed by additional t-SNARE light chains. The syntaxin family is the most conformationally dynamic group of SNAREs and appears to be the major focus of SNARE regulation. An N-terminal region of plasma membrane syntaxins has been assigned as a negative regulatory element *in vitro*. This region is absolutely required for syntaxin function *in vivo*. We now show that the required function of the N-terminal regulatory domain of the yeast plasma membrane syntaxin, Sso1p, can be circumvented when t-SNARE complex formation is made intramolecular. Our results suggest the N-terminal regulatory domain is required for efficient t-SNARE complex formation and does not recruit necessary scaffolding factors.

11

### Arf1p, Chs5p, and the ChAPs are Required for Export of Specialized Cargo from the Golgi

M. Trautwein, C. Schindler, R. Gauss, A. Spang; Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Tübingen, Germany

In *Saccharomyces cerevisiae*, the synthesis of chitin is temporally and spatially regulated through the transport of Chs3p (Chitin synthase III) to the plasma membrane in the bud neck region. Traffic of Chs3p from the TGN/early endosome to the plasma membrane requires the function of Chs5p and Chs6p. Chs6p belongs to a family of four proteins that we have named ChAPs for Chs5p-Arf1p-binding Proteins. This novel protein family is conserved throughout fungi and seemed to have arisen by three gene duplication events. We show that all ChAPs physically interact not only with Chs5p but also with the small GTPase Arf1p. A short sequence at the C-terminus of the ChAPs is required for protein function and the ability to bind to Chs5p. Disruption of two members, *Δbud7* and *Δbch1*, phenocopies a *Δchs6* or *Δchs5* deletion with respect to Chs3p transport. Moreover, the ChAPs interact with each other and form higher molecular weight complexes. In addition, they are all at least partially localized to the TGN in a Chs5p-dependent manner. Most importantly, the ChAPs interact physically with Chs3p. We propose that the ChAPs facilitate export of cargo out of the Golgi.

12

### The Role of ARF4 and ARF-GAPs in Rhodopsin Trafficking

D. Deretic,[1] L. Astuto-Gribble,[1] N. Ransom,[1] P. A. Randazzo[2]; [1]Surgery/Ophthalmology, University of New Mexico, Albuquerque, NM, [2]Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD

The small GTP-binding protein ARF4, a class II ARF, specifically recognizes and binds to the VXPX-COOH sorting motif of the light receptor rhodopsin. Using a retinal cell-free system, which reconstitutes rhodopsin trafficking in vitro, we have established that the rhodopsin-ARF4 interaction regulates the budding from the trans-Golgi network (TGN) and the incorporation of rhodopsin into the transport carriers (RTCs). RTCs are targeted to the rod outer segment (ROS), a highly specialized subcellular domain of retinal photoreceptors. To test if rhodopsin controls the ARF4-GTPase reaction by regulating the access of an ARF-GAP to ARF4, in this study we sought to identify the ARF-GAP that interacts with ARF4 in retinal photoreceptors. ARF-GAPs containing ankyrin repeats and plekstrin homology domains (AZAPs) were particularly attractive candidates given their known roles in the regulation of membrane trafficking and actin cytoskeleton. By western blotting with specific antibodies, we determined that photoreceptors express high levels of ASAP1, an AZAP that also contains the Src homology domain 3 (SH3), and has a preference for ARF1, a class I ARF, and ARF5, a class II ARF. ARAP1, an AZAP that contains a rho-GAP domain, was detected at lower levels. By confocal microscopy ASAP1 was localized to punctate structures distributed along the photoreceptor microfilaments and in the vicinity of the Golgi/TGN. ARAP1 was particularly concentrated at the outer limiting membrane where the actin cables are anchored. We are currently testing the GAP activity of ASAP1 on ARF4. Since the BAR domain of ASAP1 participates in membrane bending and fission, we are exploring if ARF4-dependent recruitment of ASAP1 to the sites of RTC budding may be the underlying mechanism for the regulation of rhodopsin trafficking by ARF4. Supported by NIH grant EY 12421.

13

### Functional Involvement of Annexin II in cAMP-Induced AQP2 Exocytosis in Renal Cells

G. Tamma, A. Strafino, F. Addabbo, M. Svelto, G. Valenti; Department of General and Environmental Physiology, University of Bari, Bari, Italy

The membrane associated protein annexin II is known to be required for the apical transport in epithelial cells. In this study we investigated the involvement of annexin II in cAMP-induced AQP2 translocation to the apical membrane. RT-PCR performed using degenerated primers revealed the presence of annexin II mRNA transcript in AQP2 expressing renal CD8 cells. Interestingly, annexin II was found in AQP2-containing vesicles immunoisolated from CD8 cells. Consistent with this observation, cell fractionation followed by Western blotting analysis showed that stimulation of CD8 cells with the cAMP-elevating agent forskolin, caused a significant increase of annexin II in the particulate fraction paralleled with a decrease in the soluble fraction. To investigate the functional involvement of annexin II in AQP2 exocytosis the fusion process between highly purified AQP2 bearing vesicles and plasma membrane was *in vitro* reconstructed and monitored by a fluorescence assay based on the dequenching of the lipophilic fluorescent probe octadecylrhodamine B-chloride (R18). We designed a peptide reproducing the N-terminal 14 aminoacids of annexin II and including binding site of the the calcium binding protein p11, a protein required for the formation of a complex with annexin necessary for tightly anchoring the protein to the cortical cytoskeleton. A control peptide having 1000 times reduced affinity for p11 was used as a control. We found that preincubation of cellular fractions with annexin II peptide strongly inhibited the fusion induced by the addition of cytosol. In contrast, the control peptide had no effect on fusion. Together these data demonstrate the association of annexin II with AQP2 containing vesicles and point to a possible functional involvement of annexin II in cAMP-induced AQP2 exocytosis in renal cells.

14

### Rab Coupling Protein (RCP) Regulates the Sorting of Membrane Proteins in Recycling Endosomes

J. J. Burden,[1] E. Schonteich,[2] G. Wilson,[2] R. Prekeris,[2] C. R. Hopkins[1]; [1]Department of Biological Sciences, Imperial College, London, United Kingdom, [2]University of Colorado Health Sciences Center, Aurora, CO

Rab coupling protein (RCP) is a class I member of the Rab11 family of interacting proteins (FIPs), a family characterised by their ability to bind Rab11 via a Rab11/25 binding domain (RBD), and to bind phospholipids via a C2 domain. Through its association with Rab11, RCP has been proposed to play a role in protein recycling. Whilst many proteins, like the transferrin receptor (TfR), have been extensively characterised and shown to faithfully follow this recycling pathway, little is known about the molecular machinery involved in regulating this trafficking route. Using RNA interference and a combination of biochemical techniques and electron microscopy, we have investigated the role that RCP plays in the intracellular trafficking of recycling proteins. By immuno-electron microscopy we have localised RCP to a tubular-vesicular compartment, that can be loaded with endocytosed transferrin-HRP. Depletion of RCP in Hela cells, was found to significantly reduce the amount of internalised transferrin in comparison to control cells, whilst the rate of internalisation of the TfR was unaffected. The reduction in transferrin uptake was accompanied by a reduction in the levels of TfR, suggesting mis-sorting of the TfR away from the recycling pathway towards the degradation pathway. To investigate this further, we used RNA interference to deplete cells of another class I FIP member, Rip11, and found that TfR recycling was not inhibited (see Wilson et al. poster). Interestingly, the co-down-regulation of RCP with Rip11 was able to rescue the effect of RCP down-regulation on the TfR, implicating Rip11 in sorting membrane proteins towards the degradative pathway. Thus, we propose that RCP plays a role in the regulation of TfR trafficking, specifically sorting the receptor from the endosomal compartment towards the plasma membrane and away from the degradative pathway.

15

### Ubiquitin Binding Proteins and Lysosomal Sorting

R. C. Piper, S. Winistorfer, J. McDermott; Physiology and Biophysics, University of Iowa, Iowa City, IA

One of the sorting signals that directs membrane proteins to lysosomes is their post-translational attachment to ubiquitin. Ubiquitin acts as a self-contained sorting signal, which acts at may intracellular locales to ultimately send membrane proteins to the lysosome. Ubiquitin works as a signal for internalization, as a TGN sorting signal and as a signal for incorporating proteins into lumenal vesicles of multivesiclular bodies. In order to understand how ubiquitin sorts proteins to the lysosome, we have focused on identifying ubiquitin-sorting receptors at the TGN and endosomes that bind cargo proteins and guide them to lysosomes. At the TGN, we find that the GGA family of clathrin binding proteins bind ubiquitin via two equivalent motifs within their GAT domains. This binding is required to direct ubiquitinated membrane proteins from the TGN directly to endosomes. We also find that several ubiquitin-binding proteins at the endosome are required for sorting into multivesicular bodies. Among these are the Vps27-Hse1 complex, the ESCRT-I complex and ESCRT-II complex. We show that physical interaction of the Vps27-Hse1 complex and ESCRT-I complex triggers a hand-off mechanism whereby ubiquitinated cargo could be transferred from one complex to the next. Interestingly, however, we find that the ubiquitin binding capacity of the ESCRT-I and ESCRT-II complexes is not required for proper sorting of ubiquitinated cargo into the MVB. Thus, we propose that the ubiquitin-binding function of these complexes is used to help Vps27-Hse1 release from cargo binding and help prevent the Vps27-Hse1 complex from being incorporated into the MVB lumen.

## Cell Biology of the Synapses (16-21)

16

### ProN-cadherin Inhibits Synapse Formation

A. G. Reines, W. Shan, A. W. Koch, D. R. Colman; BTRC, Montreal Neurological Institute, McGill University, Montreal, PQ, Canada

N-cadherin participates in the regulation of synaptic strength and stabilization. This protein is synthesized as a precursor molecule (ProN-cadherin) which has anti-adhesive properties. The Pro domain is thought to be cleaved off in the late Golgi by a furin protease, after which adhesively activated N-cadherin is directed to the cell membrane. We raised an antibody recognizing the ProN sequence, and studied the expression of ProN-cadherin in developing hippocampal cultures. We demonstrated the presence of this immature N-cadherin throughout neuronal differentiation, from 5h to 14 days in vitro. Biotinylation of the proteins on the neuronal surface revealed that a proportion of ProN-cadherin is sorted to the plasma membrane and that the mature/immature N-cadherin ratio on the surface increases as neuronal differentiation progresses. Interestingly, we found that the Pro piece is released into the culture media coincident with its decrease on the plasma membrane. To study the function of ProN-cadherin when expressed on the neuronal surface, we employed a construct in which the endogenous cleavage site was replaced by a factor Xa cleavage site. ProN-cadherin overexpression had a large impact on synapse number, measured as a decrease in the number of synaptophysin and PSD-95 puncta. The same effect was observed when the functional labeling of presynaptic boutons with the FM4-64 dye was analyzed. This effect was partially overcome when factor Xa was applied to the cultures. Our results demonstrate that anti-adhesive ProN-cadherin is expressed in neurons and sorted to the plasma membrane where it may act as a negative regulator of synapse formation. We propose that changes in the ratio of mature/immature N-cadherin on the neuronal surface might be a novel mechanism by which neurons regulate synaptic junction formation.

17

### Immaculate Connections, a Kinesin Motor, is Necessary for Presynaptic Differentiation and for the Transport of Synaptic Components

E. Pack-Chung, D. K. Dickman, T. L. Schwarz; Children's Hospital, Harvard Medical School, Boston, MA

The building blocks of synapses are present within growing axons and are recruited to sites of target cell contact. Thus, the transport of synaptic components, including the proteins of synaptic vesicles and active zones, is necessary for synaptogenesis. The identity and regulatory mechanisms of the molecular motors that move synaptic cargoes, however, remain elusive. We have identified a *Drosophila* gene (named *immaculate connections*) that is required for synaptogenesis. *immaculate connections (imac)* encodes a member of the kinesin 3 family of motors. Mutations in *imac* do not prevent axon outgrowth but prevent growth cones from transforming into synapses. At the embryonic neuromuscular junction, growth cones reach their target muscles but do not mature into synaptic boutons. Thus the development of nmj appears to be blocked just at synaptogenesis. Examination of intracellular transport revealed that the trafficking of synaptic vesicle and active zone components are blocked in

*imac*. These proteins were lacking in *imac* axons and accumulated in the cell bodies. However, post-Golgi vesicles, mitochondria, and cytoskeletal components were properly trafficked in *imac* axons. Interestingly, the absence of presynaptic differentiation in *imac* did not prevent the assembly of postsynaptic components; clustering of postsynaptic receptors and postsynaptic density elements were detected. We thus conclude that Imac is selectively required for the transport of materials for synaptogenesis and that in its absence, presynaptic differentiation fails to transpire. Our data also proves that it is distinct from the motor or motors necessary for axon outgrowth. By bringing synaptic materials into the growing axon, imac permits the rapid formation of functional synaptic connections. The coupling and uncoupling of the motor and its cargo are likely to be important in regulating the formation of synapses.

18

**HIP1 Expression is Required for Normal NMDAR Function: Implications for a Role of HIP1 in Huntington's Disease**

M. Metzler,[1] L. Gan,[1] J. Helm,[1] T. P. Wong,[2] L. Liu,[2] Y. Wang,[2] L. Liu,[1] Y. T. Wang,[2] M. R. Hayden[1]; [1]CMMT, Dept. of Medical Genetics, UBC, Vancouver, BC, Canada, [2]The Brain Research Centre, UBC, Vancouver, BC, Canada

The initial identification of the endocytic protein HIP1 (huntingtin interacting protein 1) resulted from its interaction with the polyglutamine-containing protein huntingtin that, in its polyglutamine-expanded form, causes Huntington's Disease (HD). The interaction between HIP1 and huntingtin is significantly altered following polyQ-expansion in huntingtin suggesting that HIP1 is a possible component of the pathogenic mechanism in HD. In previous studies we have shown that AMPA-induced AMPA receptor (AMPAR) trafficking is blocked in cortical neurons from HIP1 knock-out mice (Metzler et al., 2003). Here, we demonstrate a similar block in NMDA-induced AMPAR endocytosis, and hence the reduction of cell-surface AMPAR expression, in cultured hippocampal neurons from HIP1 knock-out mice. Moreover, the NMDA-induced long term potentiation of AMPAR-mediated synaptic transmission at the CA1 synapses in hippocampal brain slices from HIP1 knock-out mice is significantly reduced compared to wild-type littermates. Results from coimmunoprecipitation and GST-pulldown experiments revealed direct interaction between HIP1 and the NR2 subunit of NMDARs. Furthermore, colocalization between HIP1 and NR2-cointaining NMDARs is observed in primary hippocampal neurons. Most important for our understanding of HD, NMDA-induced excitotoxicity is blocked in neurons from HIP1 knock-out mice. Together, these data provide strong evidence that HIP1 regulates NMDAR function and that this function of HIP1 may be contributing to enhanced excitotoxicity in HD.

19

**Association of an AKAP Signaling Scaffold with Cadherin Adhesion Molecules in Neurons and Epithelial Cells**

J. A. Gorski,[1] L. L. Gomez,[1] J. D. Scott,[2] M. L. Dell'Acqua[1]; [1]Pharmacology, University of Colorado Denver Health Sciences Center, Aurora, CO, [2]Vollum Institute, Howard Hughes Medical Institute, Oregon Health Sciences University, Portland, OR

A-kinase anchoring protein (AKAP) 79/150 organizes a scaffold of PKA, PKC and protein phosphatase 2B/calcineurin that is localized to epithelial adherens junctions and the postsynaptic density of neuronal synapses. Targeting of the AKAP to these subcellular locations requires three N-terminal basic domains that bind F-actin and acidic phospholipids. Here we report a novel interaction of this targeting domain with cadherin adhesion molecules that are linked to actin through β-catenin (β-cat). Mapping the AKAP binding site in cadherins identified overlap with β-cat binding; however, no competition between AKAP and β-cat binding to cadherins was detected *in vitro*. Accordingly, AKAP79/150 exhibited polarized localization with β-cat and cadherins in epithelial cell lateral membranes, and β-cat was present in AKAP-cadherin complexes isolated from epithelial cells, cultured neurons, and rat brain synaptic membranes. Inhibition of epithelial cell cadherin adhesion induced by extracellular calcium switch and inhibition of actin polymerization by treatment with LatrunculinA redistributed intact AKAP-cadherin complexes from lateral membranes to intracellular compartments. In contrast, stimulation of neuronal pathways implicated in long term depression that depolymerize postsynaptic F-actin disrupted AKAP-cadherin interactions and resulted in loss of the AKAP, but not cadherins, from synapses. This neuronal regulation of AKAP79/150 targeting to cadherins may be important in functional and structural synaptic modifications underlying plasticity.

20

**Structural Plasticity with Preserved Topology in a Postsynaptic Protein Network**

T. A. Blanpied,[1] M. D. Ehlers[2]; [1]Physiology, University of Maryland School of Medicine, Baltimore, MD, [2]Neurobiology, Cell Biology, Pharmacology and Cancer Biology, Howard Hughes Medical Institute and Duke University Medical Center, Durham, NC

Multiprotein complexes form structural networks to mediate diverse cellular events including adhesion, signaling, nuclear transport, and intercellular communication. The function of such interconnected protein machines is determined by the spatial positioning and dynamic rearrangements of individual components within the complex. The postsynaptic density (PSD) is a prominent example of such networks. The PSD positions neurotransmitter receptors across from presynaptic sites of neurotransmitter release and links postsynaptic receptors with intracellular signaling cascades. Nearly all molecular theories of learning postulate morphological and molecular alteration of the PSD during plasticity at excitatory synapses. However, despite recent documentation of rapid mobility of receptors near the PSD, there is little known about dynamic behavior of core PSD constituents in the complex. To probe the structure of living PSDs, we have measured their internal dynamics using high-resolution imaging analyses. These experiments indicate that the PSD is assembled as a flexible yet topologically stable matrix on which enduring changes in function can be rapidly encoded. Exchange, addition, or removal of PSD elements can occur at independent matrix coordinates, providing a molecular map for organizing synaptic nanoarchitecture.

21

**Two Types of Endocytic Intermediates at the Periactive Zone of a Central Synapse**

A. Sundborger, N. Tomilin, O. Shupliakov; Neuroscience, Karolinska Institutet, Stockholm, Sweden

Dynamin is the GTPase implicated in fission of vesicles from the plasma membrane. In synapses it is an important component of the protein complex responsible for detaching clathrin vesicles from the presynaptic membrane. It has been shown in *in vitro* studies with isolated membranes and with synaptosomes that GTPγS blocks fission resulting in the accumulation of clathrin-coated pits with elongated necks decorated with dynamin-containing spirals. To visualize dynamin-dependent endocytic intermediates in an intact synapse we microinjected GTPγS into living giant axons in lamprey and studied them using electron microscopy. GTPγS did not alter morphology of synapses at rest. Stimulation of

microinjected axons with action potentials at 5 Hz induced a reduction in the number of synaptic vesicles at active zones and the appearance of numerous endocytic intermediates at periactive zones. Two different classes of intermedients were found at periactive zones: clathrin-coated pits with elongated necks, and membrane invaginations. Coated pits were present only on some of these membrane invaginations. Both were connected to the presynaptic membrane. Spiral-like structures were found at sites of these membrane connections. To investigate if these spirals contain dynamin, axons microinjected with GTPγS we cut along the longitudinal axis and stained with anti-dynamin (DG-1) antibodies using a pre-embedding immunogold technique. An accumulation of gold particles occurred at necks of clathrin-coated pits and at sites of connection of membrane invaginations to the presynaptic membrane. Our results show that two endocytic intermediates, clathrin-dependent and clathrin-independent, are formed at periactive zones during synaptic activity. Thus, in addition to the clathrin mechanism, bulk retrieval of large membrane compartments may occur in intact synapses during neurotransmitter release.

## Differentiation & Cancer (22-27)

22

### Understanding Wnt Signaling in Development and Disease

X. He[1,2]; [1]Children's Hospital, Boston, MA, [2]Harvard Medical School, Boston, MA

Wnt signaling is essential for development and tissue homeostasis. Disruption of Wnt signal transduction causes abnormal embryogenesis and cancers. Using a combination of molecular, biochemical and embryological techniques, we have focused on the mechanism of Wnt signaling in Xenopus embryo development and human cancer. We are particularly interested in how the Wnt receptor complex transduces Wnt signal across the plasma membrane, how the Wnt receptor complex specifies distinct transduction pathways to govern different aspects of embryogenesis, and the molecular composition and logic of these transduction pathways. Protein phosphorylation is pivotal for Wnt signaling. We have characterized two key phosphorylation events in the canonical Wnt/beta-catenin pathway. One is phosphorylation of the Wnt coreceptor, LDL receptor related protein 6 (LRP6). This phosphorylation leads to LRP6 activation and the initiation of Wnt signal transduction. The other is beta-catenin phosphorylation, which results in beta-catenin degradation and is inhibited upon Wnt signaling. **1). The mechanism of phosphorylation and activation of the Wnt coreceptor LRP6.** We have shown that Wnt induces LRP6 phosphorylation at PPPS/TP motifs, and this phosphorylation is necessary and sufficient to trigger Wnt signaling. We have generated antibodies that specifically recognize phosphorylated LRP6. These antibodies are useful tools for detection of Wnt signaling activation in vivo and for identification of kinases involved in LRP6 phosphorylation. I will discuss our recent progress in studying Wnt-induced LRP6 phosphorylation and identifying LRP6 kinases. **2). The mechanism of phosphorylation and degradation of beta-catenin.** We have demonstrated that two kinases, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), sequentially phosphorylate beta-catenin in a protein complex assembled by the scaffolding protein Axin. I will discuss how Wnt receptor activation may lead to inhibition of beta-catenin phosphorylation, and our attempt to identify other key molecules via in vitro reconstitution.

23

### p21WAF1/Cip1 Mediates Notch1-dependent Suppression of Wnt Expression and Signaling

V. Devgan,[1] C. Mammucari,[2] S. E. Millar,[3] C. Brisken,[4] G. P. Dotto[2]; [1]Cutaneous Biology Research Center, Massachusetts General Hospital, Charlestown, MA, [2]Department of Biochemistry, Lausanne University, Epalinges, Switzerland, [3]Department of Dermatology, University of Pennsylvania Medical School, Philadelphia, PA, [4]Swiss Cancer Research Institute, Epalinges, Switzerland

Besides controlling cyclin/CDK and PCNA activities, $p21^{WAF1/Cip1}$ can directly bind transcription factors and co-activators, modulating their functions. However, the biological significance of these findings has not been established. In keratinocytes, p21 is a direct downstream target of Notch1 activation, and loss of either p21 or Notch1 expands keratinocyte stem cell populations and facilitates tumor development. The tumor suppressor function of Notch1 has been associated with negative regulation of β-catenin signaling, through an unknown mechanism. We show here that Notch1 activation down-regulates β-catenin signaling through suppression of Wnts gene expression, and that p21 is a key mediator of this down-modulation. p21 suppresses Wnts expression independently of the cell cycle, while lack of p21 prevents Notch-dependent suppression of Wnt gene expression and results, both in cultured keratinocytes and in the intact skin *in vivo*, in increased Wnt 4 expression. More specifically, p21 associates with the E2F-1 transcription factor at the *Wnt4* promoter and causes curtailed recruitment of c-Myc and p300, and histone hypoacetylation at this promoter. Thus, p21 functions as a mediator of the negative effect of Notch1 activation on Wnt signaling, by specific down-modulation of Wnt gene expression at the transcription-chromatin level and independently of cell cycle.

24

### Gata-3 is a Critical Regulator of Differentiation in the Mammary Gland and Breast Cancer

H. Kouros-Mehr, Z. Werb; Anatomy, UCSF, San Francisco, CA

A series of breast cancer microarray studies have shown that the transcription factor GATA-3 is strongly correlated with Estrogen Receptor (ER) status, tumor grade and survival. We show here that GATA-3 plays a fundamental role in maintaining the luminal epithelial cell fate in the mammary gland and in breast cancer. We initially identified GATA-3 through a microarray screen as the most highly expressed transcription factor in the mammary gland. Immunostaining revealed that GATA-3 is expressed exclusively in all luminal progenitors and differentiated luminal cells. To determine the function of GATA-3, we crossed floxed GATA-3 mice with MMTV-Cre and WAP-rtTA-Cre lines. Homozygous floxed GATA-3 mice carrying MMTV-Cre displayed runting, progressive alopecia, and a highly defective mammary gland. The mammary glands displayed a near lack of epithelium due to an inability to form terminal end buds. To more closely analyze the function of GATA-3, we crossed the floxed mice with the Tet-inducible Cre line WAP-rtTA-Cre. After short-term (72 hr) administration of doxycycline to adult mice, GATA-3 null luminal cells displayed loss of basal polarity and died within the ductal lumen as single cells. To determine how GATA-3 affects breast cancer progression, we used the PyMT mouse model of breast cancer. We transplanted GFP+ hyperplasias into syngeneic mice to determine when malignant conversion occurred. We found that loss of GATA-3 strongly correlated with loss of tumor differentiation, the progression from adenoma to early carcinoma, and the onset of tumor dissemination into distant sites. Furthermore, overexpression of GATA-3 in primary PyMT tumors was sufficient to induce elements of tumor differentiation. This work suggests that GATA-3 specifies and maintains luminal cell differentiation and suggests that loss of cell fate is a critical event in the malignant progression of breast cancer.

25

### Six1 Promotes Rhabdomyosarcoma Metastasis through Ezrin

Y. Yu; National Cancer Institute, Bethesda, MD

Rhabdomyosarcoma (RMS), thought to arise from skeletal muscle precursors, is the most common soft-tissue sarcoma in children. Despite recent improvements in long-term survival rates, about one third of RMS patients continue to experience relapses, and the majority of these patients die from disseminated metastatic disease. This is due in large part to our rudimentary knowledge of molecular pathways that dictate metastatic potential. Previously, we employed cDNA microarray analysis of RMS cell lines derived from a mouse RMS model, based on deficiency in Ink4a/Arf and deregulation of the hepatocyte growth factor/scatter factor (HGF/SF)-Met signal transduction pathway, to identify a set of genes whose expression was significantly different between highly and poorly metastatic cells. We discovered that the cytoskeletal organizer Ezrin and the developmental homeoprotein Six1 were key metastatic regulators (Nature Medicine, 10, 175, 2004). We have recently found that Ezrin is a direct transcriptional target of Six1; the Six1 transcription factor can bind to the promoter of the Ezrin gene and regulate its expression. Overexpression of Six1 in RMS cells increases Ezrin protein level, whereas Six1 knockdown by siRNA decreases Ezrin protein. Moreover, blocking Ezrin in RMS cells through siRNA can abrogate the metastatic potential of Six1 in vivo. Therefore, Six1 stimulates metastasis of RMS cells through Ezrin. These data show that Six1 and Ezrin are critical regulators of metastasis in RMS, and provide new mechanistic and therapeutic insights into this pediatric cancer.

26

### Differentiative State Dependent Transcription and Tumorigenesis Elicited by *MYC*

C. Wu,[1] D. Sahoo,[2] C. Arvanitis,[1] N. Bradon,[1] D. Dill,[2] D. W. Felsher[1]; [1]Department of Medicine, Division of Oncology, Stanford University, Stanford, CA, [2]Department of Computer Science, Stanford University, Stanford, CA

Cancer is commonly associated with the malignant expansion of immature cells. The *MYC* oncogene is a transcription factor that when overexpressed is thought to induce tumorigenesis by causing inappropriate gene expression resulting in cellular proliferation, growth and blocking differentiation. Many *MYC* target genes have been identified; however, which *MYC* target genes are actually required for initiating or maintaining a tumor phenotype is unknown. We have established a transgenic mouse model for conditionally regulating *MYC* expression. By using this system, we have found that even a brief inactivation of the *MYC* oncogene can result in the sustained loss of a neoplastic phenotype. We demonstrated that upon *MYC* inactivation immature osteogenic sarcoma cells differentiated into mature osteocytes and formed bone. Surprisingly, *MYC* reactivation failed to restore a neoplastic phenotype, and instead induced the now differentiated tumor cells to undergo apoptosis. Our results suggest the possibility that the inactivation of the *MYC* oncogene may cause epigenetic changes in tumor cells such that they lose the ability to be tumor cells. We hypothesized that upon differentiation, the tumor cells undergo chromatin remodeling which prevents *MYC* from binding to the promoter regions of the genes that are required to induce or sustain tumorigenesis. Indeed, we have shown by cDNA microarrays that upon *MYC* inactivation that there are a multitude of changes in gene expression and that upon *MYC* reactivation many of these changes are not restored to their previous state. Our results suggest that the transcriptional program elicited by *MYC* depends upon the differentiative state and this may define the ability of *MYC* to induce tumorigenesis.

27

### Multiple Signaling Pathways Control the Maintenance and Differentiation of Somatic Stem Cells in the *Drosophila* Ovary

T. Xie, D. Kirilly, X. Song; Stowers Institute for Medical Research, Kansas City, MO

Stem cells are responsible for replenishing lost cells in adult tissues throughout lifetime. One of the challenges in the filed of stem cells is to identify extrinsic factors that control stem cell self-renewal, proliferation and differentiation. The *Drosophila* ovarian somatic stem cells (SSCs) are responsible for producing epithelial cell-like follicle cells that surround differentiated germ cells in egg chambers, providing a nice model system to study epithelial stem cell regulation. In this system, Hedgehog has been shown by others to be important for maintaining ovarian SSCs (King et al., 2001; Zhang and Kalderon, 2001). Recently, we have shown that Wingless signaling is also essential for maintaining SSCs and that its hyperactive signaling causes abnormal follicle cell differentiation and proliferation (Song and Xie, 2003). Here, we report our recent studies on the roles of BMP and Jak-Stat signaling in SSC regulation by examining marked SSCs defective in these pathways. The marked SSC clones defective in the BMP or Jak-Stat pathway are lost rapidly in comparison with marked wild-type control SSC clones, while hyperactive signaling for one of these pathways disrupts normal differentiation of SSC progeny, causing abnormal follicle cell proliferation. Interestingly, Shh, Wnt, BMP and JAK-STAT signaling pathways have been also shown to be involved in cancer formation and stem cell regulation in mammals. The detailed analysis of the roles of these pathways in SSC self-renewal, proliferation and differentiation will be presented.

## Extracellular Matrix & Signaling (28-33)

28

### Signaling by Thrombospondin-1

J. C. Adams, R. Chakravarti, Y. Hashimoto, M. Valiyaveettil; Department of Cell Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH

Thrombospondins (TSPs) are conserved, extracellular, multimeric, calcium-binding glycoproteins of animals. In mammals, TSPs function within ECM, at cell-surfaces, and as bridging molecules in cell-cell interactions to regulate cell adhesion, migration, angiogenesis, connective tissue organisation and other cell behaviors. The TSPs of invertebrates may function in development and in specialised ECMs (for TSP reviews, see Int. J. Biochem. and Cell Biol. 36, issue 6, June 2004). In all TSPs, the most highly-conserved region is the C-terminal half, comprising EGF domains, type 3 repeats and a C-terminal, L-lectin-like domain. Our interest is in the molecular processes by which TSP-1 induces cell adhesion, actin organisation and motility. For normal mesenchymal cells, spreading on intact TSP-1 signals the formation of protrusions in which F-actin is bundled by the cross-linking protein, fascin. Cell attachment is mediated by the last three type 3 repeats and L-lectin domain of TSP-1, yet native trimeric assembly is needed to support cytoskeletal assembly and migration. Of the transmembrane adhesion receptors that bind TSP-1, syndecan-1 has a key role in transducing lamellipodial spreading and actin-bundling by fascin. We have established that the V region of syndecan-1

cytoplasmic domain is critical for this activity. Whereas TSP-1-adherent cells maintain the actin-bundling form of fascin which is non-phosphorylated at residue S39, other ECM components such as fibronectin signal fascin phosphorylation through integrin-dependent activation of protein kinase C. Phosphorylated fascin associates with the regulatory domain of protein kinase Calpha and this complex acts to modulate the balance between focal adhesions and protrusions, resulting in alterations to cell motility behavior. These findings demonstrate distinct signaling activities of TSP-1 and uncover a mechanism for intersection with other signaling processes activated by complex ECM.

29

**Thrombospondin 1 induced Akt activation and anoikis resistance**

C. A. Elzie, J. Murphy-Ullrich; Department of Pathology, University of Alabama, Birmingham, AL

Thrombospondin 1 (TSP1) is a matricellular protein that is up-regulated in areas of active tissue remodeling. We previously showed that amino acids 17-35 of TSP1 or a peptide comprised of this sequence (hep I) binds to the receptor co-complex of cell surface calreticulin and LDL receptor-related protein (LRP1), stimulating focal adhesion disassembly and cell motility through activation of PI3K, FAK, ERK and downregulation of Rho signaling. We now show that hep I initiates cell survival signals in mouse embryonic fibroblasts and prevents apoptosis caused by a loss of cell-matrix signals (anoikis). Anoikis is mimicked in cell culture by plating cells on poly-HEMA, which does not support adhesion. Cells on poly-HEMA show increased cleavage of PARP and annexin V staining, indicative of cell death. Addition of hep I to cells reduces PARP cleavage and annexin V staining. Calreticulin is also highly up-regulated in cells plated on poly-HEMA. Since hep I activates PI3K, we investigated whether hep I activates Akt and whether Akt is important for hep I rescue from anoikis. Hep I, the heparin binding domain of TSP1 (Nco1) and TSP1 transiently activate Akt. TSP1/hep I binding to calreticulin is important for Akt activation since a peptide that blocks TSP1/hep I binding to calreticulin and calreticulin null fibroblasts are unable to activate Akt by TSP1, but not by insulin. Adding exogenous calreticulin back to the cell surface restores TSP1-induced Akt activation. An inhibitor of ligand binding to LRP1, RAP, also blocks Akt activation by TSP1. Taken together, the data show that TSP1 binding to calreticulin/LRP1 through its hep I sequence, activates anti-apoptotic signaling. These data suggest that TSP1 plays a critical role in cellular responses to injury and tissue remodeling.

30

**Extracellular Matrix Protein CCN1 (CYR61) Induces Apoptosis in Fibroblasts**

V. Todorovic, C. C. Chen, N. Hay, L. F. Lau; Biochemistry and Molecular Genetics, University of Illinois, Chicago, IL

Integrin-mediated cell adhesion to extracellular matrix proteins is known to promote cell survival, whereas detachment from the matrix can cause rapid apoptotic death in some cell types. Contrary to this paradigm, we show that fibroblast adhesion to the angiogenic matrix protein CCN1 (CYR61) induces apoptosis, whereas endothelial cell adhesion to CCN1 promotes cell suvival. CCN1 induces fibroblast apoptosis through its adhesion receptors, integrin $\alpha_6\beta_1$ and the heparan sulfate proteoglycan (HSPGs) syndecan-4, triggering the p53-dependent activation of Bax to render cytochrome c release and activation of caspases 9 and 3. Neither caspase 8 activity nor *de novo* transcription or translation is required for this process. These results show, for the first time, that cellular interaction with a specific matrix protein can either induce or suppress apoptosis in a cell type-specific manner, and that integrin $\alpha_6\beta_1$-HSPGs can function as receptors to induce p53-dependent apoptosis.

31

**Extracellular Cues Influence Fibronectin Alternative Splicing Through an Intrincate Network of Signaling Pathways**

F. Pelisch, M. Blaustein, L. Quadrana, A. Srebrow; Departamento de Fisiología, Biología Molecular y Celular - Facultad de Ciencias Exactas y Naturales, Laboratorio de Fisiología y Biología Molecular - IFIBYNE, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

Alternative pre-mRNA splicing is the most important source of protein diversity in vertebrates. Regulation of this process by extracellular cues represents a key event in the control of gene expression. We use mouse mammary cell lines and the fibronectin gene to study the linkage between the cellular microenvironment and the splicing machinery. We found that a laminin-rich basement membrane down-regulates the inclusion of two fibronectin alternative regions, EDA and IIICS, through a JNK-dependent pathway in epithelial cells. We propose that dephosphorylation of ERK is involved in this regulatory process. By contrast, soluble factors from a mammary mesenchymal cell-conditioned medium as well as different growth factors up-regulate their inclusion via PI 3-kinase. The laminin-rich basement membrane blocks the effect of the mammary mesenchymal cell-conditioned medium, extending the already proposed antagonism between these two signaling pathways to the field of alternative splicing. These results highlight the fact that the splicing pattern of a single transcript is the read out of an intricate network of different signaling cascades. SR proteins have emerged recently as much more than splicing regulators and their activity has been associated to different steps along mRNA metabolism. We found that the signaling pathway triggered by soluble factors not only affect mRNA splicing but also alter translation of reporter mRNAs containing a fibronectin EDA exonic splicing enhancer. These effects on splicing and translation are dependent on SR proteins and can be duplicated by over-expressing a constitutively active AKT, a downstream target of PI 3-kinase. These latter results show how SR protein activity is modified in response to extracellular cues leading to a concerted regulation of splicing and translation.

32

**Laminin-5 Beta 3 Chain Promotes Epidermal Carcinogenesis Through Type VII Collagen Binding and Pi3k Activation**

N. Sakai, E. A. Waterman, N. T. Nguyen, B. A. J. Horst, D. P. Veitch, C. N. Dey, S. Ortiz-Urda, P. A. Khavari, M. P. Marinkovich; Epithelial Biology, Stanford University, Stanford, CA

Laminin-5 plays an essential role in epidermal carcinogenesis but the underlying mechanisms remain unclear. To further address this we retrovirally expressed two laminin beta-3 chain truncations, termed Tr1 (deletion domain VI) and Tr2 (deletion domains VI-V-III), in laminin beta-3 negative epidermolysis bullosa keratinocytes. Tr1/Tr2 laminins were properly assembled, expressed at wild type levels and deposited into keratinocyte extracellular matrix. Tr1/Tr2-expressing keratinocytes displayed normal migration and spreading but showed decreased stable adhesion as measured by trypsin detachment assay and showed abnormal localization of actin filaments and focal adhesion components to hemidesmosomes. Despite these adhesive defects, Tr1 cells transformed with Ras and IkBa produced SCC-like tumors after SQ injection to nude mice and showed Matrigel invasion equal to control transformed keratinocytes. In contrast, Ras/IkBa transformed Tr2 cells showed a complete

inability to form tumors or invade Matrigel. Transformed Tr2 cells displayed decreased PI3-kinase activity compared to transformed Tr1 and wild type cells. Retroviral transfer of active PI3-kinase cDNA restored normal matrigel invasion to Tr2 cells. Moreover, PI3-kinase expression completely restored tumorigenesis and tissue invasion to transformed Tr2 cells after SQ injection to nude mice. Transformed type VII collagen null keratinocytes were also shown to have deficient PI3-kinase and invasive activity, which was restored by retroviral transfer of PI3-kinase cDNA. Purified Tr1 laminin (lacking domain VI) bound to collagen VII by solid phase assay in a normal dose-dependent manner, whereas purified Tr2 laminin (lacking domain VI-V-III) failed to bind type VII collagen. These studies suggest that domain VI mediates stable adhesion involving transition of focal adhesions to hemidesmosomes, but this process is dispensable for tumorigenesis. Instead it is the binding of laminin beta-3 domain V-III to type VII collagen which promotes epidermal carcinogenesis through PI3-kinase activation.

33

**CD98hc (SLC3A2) Supports Fibronectin Matrix Assembly by Capacitating Outside-In Integrin Signaling**

C. C. Feral,[1] A. Zijlstra,[2] G. Prager,[1] M. Slepak,[1] M. H. Ginsberg[1]; [1]Dpt. of Medicine, University of California San Diego, La Jolla, CA, [2]Dpt. of Cell Biology, The Scripps Research Institute, La Jolla, CA

The cell-mediated assembly of soluble fibronectin into a fibrillar matrix is essential for vertebrate development. The initiation of matrix assembly involves the binding of soluble fibronectin to high affinity integrins, such as integrin α5β1, which transmit cellular contractile forces to the fibronectin, leading to exposure of matrix assembly sites. In addition to transmitting force to fibronectin, integrins can initiate intracellular biochemical signals, such as activation of Src kinases and Focal Adhesion Kinase (FAK). We have recently found that CD98 heterodimers comprised of a common heavy chain (CD98hc, SLC3A2), and one of several light chains, interact with integrins via the CD98hc moiety and mediate the integrin-dependent activation of FAK and phosphorylation of several Src substrates. We disrupted the CD98hc gene in embryonic fibroblasts and we report here that CD98hc is required for fibronectin matrix assembly, but not for fibronectin biosynthesis. The loss of matrix assembly is not due reduced integrin affinity because the CD98hc null fibroblasts bound soluble fibronectin cell binding domain with the same affinity as wild-type cells and because enforced activation of integrin α5β1 with a monoclonal antibody did not rescue the assembly. CD98hc mutants that restore integrin-dependent biochemical signaling, rescued matrix assembly. In contrast, mutants that fail to associate with integrins or promote integrin signaling failed to reconstitute matrix assembly. Thus, outside in signals, mediated by an integrin-CD98 complex, lead to activation of FAK and Src and promote fibronectin matrix assembly.

## Formins & Arp2/3: Regulators of Actin (34-39)

34

**A Novel Arp2/3-Activating Protein that Interacts with Dynamic Endomembrane Compartments and Microtubules**

N. J. Webb, E. A. Znameroski, M. D. Welch; Molecular & Cell Biology, University of California, Berkeley, Berkeley, CA

The Arp2/3 complex is a central actin nucleating and organizing factor that functions in a variety of processes including lamellipodia protrusion, podosome formation and endocytosis. The activities of the Arp2/3 complex are regulated by a class of proteins called nucleation-promoting factors (NPFs). Mammalian cells express several well-studied NPFs including WASP, N-WASP, and Scar/WAVE1-3, each of which may play a distinct function in regulating actin nucleation in the cell. Given the diversity of NPFs and their important role in regulating actin dynamics, we sought to identify additional uncharacterized members of this protein family. Here we report the identification of a novel class of NPFs comprised of two proteins, KIAA1971 and JMY. Each contains a C-terminal WCA domain that is a conserved feature of NPFs, a central region predicted to form coiled-coils, and an N-terminal domain of unknown function. Our studies show that the recombinant full-length KIAA1971 protein and its WCA domain activate the Arp2/3 complex in vitro, confirming that it is an NPF. To evaluate its cellular function, we analyzed its localization in stably-transfected cells. Interestingly, GFP-KIAA1971 localizes to rapidly moving internal membranes, suggesting a role in endomembrane dynamics. Drugs that perturb actin and microtubule dynamics interfere with the movement of GFP-labeled membranes, implying that both cytoskeletal networks play a role in motility. Surprisingly, we also find that GFP-KIAA1971 colocalizes with microtubules in transiently-transfected cells that overexpress the protein. We mapped the domain of KIAA1971 responsible for microtubule localization to the central coiled-coil region using GFP-tagged N- and C-terminal truncations. Overexpression of this domain causes the bundling and stabilization of microtubules, hinting at a role in microtubule function. In summary, our studies suggest a novel role for NPFs in integrating actin and microtubules with the regulation of endomembrane dynamics.

35

**Reconstitution of N-wasp-dependent Vesicle Motility from Pure Components: Spontaneous "symmetry Breaking" via Stochastic, Localized Activation of the Cdc42/rhogdi Complex**

D. T. Wong, C. Co, J. Taunton; University of California, San Francisco, San Francisco, CA

Actin polymerization at the plasma membrane drives cell motility. A minimal motility medium consisting of 4 purified proteins (actin, Arp2/3 complex, cofilin, and capping protein) has provided a simplified model system with which to study the mechanism of actin-based force generation. Plastic beads uniformly coated with Arp2/3 activators such as ActA or N-WASP break symmetry slowly, by assembling a symmetrical actin gel that eventually ruptures and transforms into an asymmetric, force-generating comet tail. By contrast, in cells and cytoplasmic extracts membrane-bound organelles appear to break symmetry instantaneously. The biophysical mechanism by which this occurs is unknown. Using purified proteins and lipid bilayer-coated glass microspheres, we have developed a novel reconstitution system that integrates guanine nucleotide exchange factor (GEF)-dependent activation of the Cdc42/RhoGDI complex with actin nucleation promoted by N-WASP and the Arp2/3 complex. Upon GEF-mediated activation at the membrane surface, prenylated Cdc42 is released from its soluble inhibitor, RhoGDI. Stochastic, membrane-localized activation of Cdc42 and N-WASP in the motility medium leads to the instantaneous assembly of an asymmetric comet tail, with no evidence for a symmetrical actin gel. In contrast, when lipid-coated beads are pre-activated with Cdc42 and N-WASP and then placed in the motility medium, a symmetrical actin gel forms, similar to plastic beads. Our reconstitution system provides insights into the mechanism by which a localized signal at the membrane can rapidly generate protrusive motility.

36

**Mechanisms of Actin Bundle Formation by Mammalian Formins**

E. S. Harris,[1] I. Rouiller,[2] D. Hanein,[2] H. Higgs[1]; [1]Department of Biochemistry, Dartmouth Medical School, Hanover, NH, [2]Burnham Institute, La Jolla, CA

Formins are a conserved family of actin-associated proteins, with many eukaryotes expressing multiple isoforms. The biochemical properties of several yeast and mammalian formin FH2 domains have been studied in vitro. Formin FH2 domains have the following effects on actin dynamics: 1) accelerating nucleation rate, 2) altering elongation/depolymerization rates, and 3) antagonizing barbed end capping by capping proteins. The potency of each effect varies between formins, reflecting quantitative differences in an apparently similar mechanism centered around barbed end binding by FH2 domains. We have found that a subset of mammalian formins have a fourth activity, filament side binding and bundling. FH2 domain-containing constructs of FRL1, FRL2, and mDia2 can bundle actin filaments, while mDia1's FH2 domain cannot. Filament side binding and bundling is dependent only on the FH2 domain. Filaments bundled by formins are arranged in a predominately anti-parallel orientation. Our results suggest that individual formins may have different mechanisms for bundling. mDia2's bundling activity is not competitive with barbed end binding. Additionally, mutation of a conserved residue in the mDia2 FH2 domain that faces the inside of the FH2 "donut" drastically reduces mDia2's affinity for the barbed end, but has no effect on bundling ability. This suggests mDia2 may bind filament sides through interactions that involve residues on the outside of the FH2 dimer, and are different from residues used for barbed end binding. In contrast, FRL1's bundling activity is competitive with barbed end binding suggesting it may occur through residues on the inner face of the FH2 dimer.

37

**Dynamics of the Formin For3p and Actin Cables in Fission Yeast**

S. G. Martin, F. Chang; Department of Microbiology, Columbia University, New York, NY

How formins are localised and regulated to form dynamic actin structures at the right place *in vivo* is still poorly understood. In the fission yeast *Schizosaccharomyces pombe*, the formin for3p functions at cell tips to assemble actin cables, which are thought to be dynamic bundles of short actin filaments oriented with barbed ends facing the cell tip. Using a for3p-3GFP fusion expressed at endogenous levels, we observed small dots of for3p that stream in linear paths away from the cell tip at a rate of 0.3μm/s. This rate is similar to actin polymerization rates, as measured by imaging actin cable markers. The for3p retrograde movements are dependent on actin cables and on actin binding residues within the FH2 domain of for3p. In addition, we observed anterograde movements of for3p dots towards the cell tips at a significantly faster rate (1.1μm/s), which is consistent with myo52p (myosin type V)-driven movements. Indeed, these anterograde movements are not detected in *myo52* mutants. Further, for3p and myo52p associate with each other in co-immunoprecipations. FRAP studies confirm that for3p is highly dynamic at cell tips. These studies provide a new model for actin cable formation and formin dynamics. We propose that for3p is present and active for actin assembly at the cortex for only seconds. It may then remain bound inactive on the barbed end of the actin filament, where it is carried passively by bulk movement of the actin cable away from the cell tip. Myosin-V directed transport on actin cables may contribute to targeting for3p back to the cell tips. These studies suggest the existence of a dynamic actin assembly machine at the cortex that transiently docks, activates and then releases formins.

38

**Dissecting the Role of mDia2 in Actin Dynamics in Migrating Epithelial Cells**

S. L. Gupton,[1] K. M. Eisenmann,[2] A. S. Alberts,[2] C. M. Waterman-Storer[1]; [1]Cell Biology, Scripps Research Institute, La Jolla, CA, [2]Van Andel Research Institute, Michigan State University, Grand Rapids, MI

Formins nucleate and protect actin filaments from capping, and promote polymerization from fast growing filament ends in vitro, but their role in migrating cells is unknown. Previously, we have used quantitative Fluorescent Speckle Microscopy (qFSM) to define actin-based modules mediating epithelial cell migration: the lamellipodium being the protrusive module and the lamella being the adhesive/contractile module. The actin nucleating complex Arp2/3 is active in the lamellipodium of epithelial cells, but it is unclear where formin proteins are active and how they contribute to actin cytoskeleton remodeling in these modules of migrating cells. To test the hypothesis that mDia2 is involved in actin polymerization in the lamella, we used a combination of FRET and qFSM microscopic assays along with reagents to manipulate mDia activity. To examine where mDia2 interacts with and is potentially activated by different Rho GTPases with respect to these two zones, FRET was employed by expressing CFP-GTPase and YFP-mDia2 fusion proteins to act as donor/acceptor pairs respectively. YFP-mDia2 was bound to CFP-Cdc42 in filopodia at the leading edge and at focal adhesion sites in the lamella. Consistent with a role for mDia2 at these sites, inhibition of mDia2 by microinjection of an antibody that specifically inhibits mDia2-mediated actin nucleation in vitro decreased the amounts of free filament barbed ends at focal adhesions. Expression of an activating subunit of mDia (DAD), an inhibitory subunit of mDia (FH2δN), or microinjection of the inhibitory antibody affected lamellipodium stability and the typical patterns of actin polymerization/depolymerization observed by qFSM in the lamella and lamellipodium of control cells. These changes in actin turnover and movement correlated with inhibition of cell migration, suggesting that proper spatio-temporal activity of mDia2 is necessary for proper actin organization and dynamics and efficient cell migration.

39

**Mechanism of Filopodia Formation Induced by mDia2**

C. Yang,[1] L. A. Czech,[2] S. Kojima,[2] G. G. Borisy,[2] T. M. Svitkina[1]; [1]Department of Biology, University of Pennsylvania, Philadelphia, PA, [2]Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL

Expression of GFP-tagged constitutively active mDia2(ΔGBD) mutant induced filopodia-like protrusions in B16F1 cells with mDia2(ΔGBD) localizing to their tips. These processes frequently had an unusual club-like shape with a thick bundle of unbranched actin filaments in the distal domains, which tapered towards the rear so that a smaller number of filaments formed a thinner bundle in the stalk. Many free pointed ends could be seen in the region of bundle tapering. Depolymerization from these ends apparently was responsible for the club-like shape of mDia2(ΔGBD)-induced filopodia. Kinetic studies showed that initiation of mDia2(ΔGBD)-induced filopodia frequently occurred by sudden appearance of a GFP-positive dot at the cell edge or dorsal surface. While the dot grew in intensity, a filopodial bundle developed at this site. Fusion of two or more mDia2(ΔGBD)-induced filopodia led to formation of larger club-like protrusions. Nascent mDia2(ΔGBD)-induced filopodia contained tapered actin bundles with multiple free pointed ends at the rear. Such structure contrasts the lambda-like shape of normal nascent filopodia, where actin

filament pointed ends are engaged in branch formation in the surrounding network. mDia2(ΔGBD) also localized along the leading edge of some lamellipodia. Such lamellipodia had unusual actin organization and contained numerous long unbranched filaments with free pointed ends. Over time, they usually split into several filopodia, apparently, due to initiation of bundling. Ena/VASP proteins were not required for filopodia formation by mDia2(ΔGBD) as shown by transfection of $MV^{D7}$ fibroblasts lacking all endogenous Ena/VASP proteins. Moreover, over-expressed mDia2(ΔGBD) displaced endogenous or co-expressed VASP from filopodial tips. Together, these data show that constitutively active mDia2 is recruited to the membrane where it induces filopodia by nucleating actin filaments and subsequently protecting their barbed ends from capping. Supported by NIH grants GM 62431 (GGB) and GM 070898 (TMS).

## Nuclear Envelope Functions (40-45)

40

### Insight into the Nucleocytoplasmic Transport Mechanism from Novel Assays for FG-Repeat Requirements and mRNA Export Kinetics

L. J. Terry, S. R. Wente; Department of Cell & Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

Nucleocytoplasmic transport requires interactions between transport receptors, which accompany cargo during transport, and nucleoporins (Nups), which comprise the nuclear pore complex (NPC). Nearly half of the ~30 Nups contain domains of phenylalanine-glycine (FG) repeats, and these FG-Nups constitute critical binding sites for intermediate points in nucleocytoplasmic transport. Using genetic strategies in *Saccharomyces cerevisiae* we generated a collection of strains depleted of higher-order combinations of FG-repeat domains (Strawn *et al.* Nature Cell Biology 2004) and determined that the five asymmetrically distributed FG-repeat domains, creating strain ΔNΔC, are unnecessary for viability and bulk transport. However, symmetrically distributed FG-repeat domains are required in discrete combinations. Although there is a growing body of evidence on the interactions between transport receptors and FG-Nups, the precise mechanism of nucleocytoplasmic transport remains unknown. To gain a better understanding of the transport mechanism, we have utilized FG-repeat deletion strains to study the mRNA transport receptor Mex67p/TAP/Nxf1 and several members of the karyopherin-B family of transport receptors. We have taken advantage of the ΔNΔC strain background to analyze the roles of the symmetric FG-Nups. Further deletions in the ΔNΔC strain resulted in viable more minimal strains, which have served as a powerful tool to identify symmetric FG-repeat domains critical to specific transport pathways while preventing the possibility of compensation by asymmetric FG-repeats. We have screened FG-repeat deletion strains by *in situ* hybridization for poly(A)RNA and have developed an assay to detect the rate of recruitment of Mex67p to NPCs as a measure for mRNA export. Notably, specific FG-repeat deletions cause defects in poly(A)RNA export and impair the recruitment of Mex67p to the NPC. These studies provide insight into the complex series of interactions between FG-Nups and transport receptors required for efficient nucleocytoplasmic transport.

41

### Pml39, a Novel Nuclear-Pore Associated Protein Involved in mRNA Surveillance in Budding Yeast

B. Palancade,[1] M. Zuccolo,[1] S. Loeillet,[2] A. Nicolas,[2] V. Doye[1]; [1]Section Recherche, UMR 144 CNRS - Institut Curie, Paris, France, [2]Section Recherche, UMR 7147 CNRS - Institut Curie, Paris, France

In *S. cerevisiae*, perinuclear improper mRNP retention involves the Mlp1-2 nucleoporins. Here we describe a new yeast protein belonging to this pathway, Pml39 (for pre-mRNA leakage, 39 kDa). *PML39* was identified on the basis of a strong and specific genetic interaction with genes encoding nucleoporins of the Nup84 nuclear pore subcomplex. Like Mlp1 and Mlp2, Pml39 is associated with a subset of nuclear pore complexes (NPCs) facing the chromatin, opposite to the nucleolus. As further established by mislocalization of the GFP fusion and two-hybrid analysis, Pml39 is mainly docked at NPCs through interaction with Mlp1 and Mlp2 N-terminal domains. Functional studies revealed that the *PML39* deletion induces a specific leakage of unspliced mRNAs out of the nucleus, that is not additive to the one exhibited by *mlp1Δ*. In addition, overexpression of a *PML39-GFP* fusion leads to a specific trapping within discrete nuclear domains of mRNAs transcribed from an intron-containing reporter and of the hnRNP Nab2. In a *nup60Δ* mutant, Pml39 is mislocalized together with Mlp1 and Mlp2 in intranuclear foci that also recruit Nab2. Moreover, the temperature-sensitive phenotypes of mutants of mRNPs components such as *GFP-yra1-8* and *ΔN-nab2* were partially rescued by the *pml39* deletion. Thus, *pml39* deletion bypasses the requirement for normally assembled mRNPs, demonstrating that the function of the Pml39 protein is to retain improper mRNPs in the nucleus. Pml39 appears as an upstream effector of the Mlp1/2 pathway, required for the quality control steps occuring prior to the export of mRNAs out of the nucleus.

42

### The Nucleoporin Gp210 is Required for Nuclear Envelope Breakdown

W. Antonin,[1] V. Galy,[2] I. W. Mattaj[1]; [1]Gene Expression, European Molecular Biology Laboratory, Heidelberg, Germany, [2]Unité de Biologie Cellulaire du Noyau, Institut Pasteur, Paris, France

In higher eukaryotes the nuclear envelope is a dynamic structure during cell cycle: It breaks down at beginning of mitosis and reforms around the separated chromatin at its end. During nuclear envelope breakdown (NEBD) nuclear pore complexes are disassembled into nucleoporins being present during mitosis mostly in soluble form. The membranes are at least in mammals reabsorbed into the endoplasmic reticulum and the lamina, which is underlying the inner nuclear membrane in interphase, is disassembled. Although these processes have been known for a long time the molecular mechanisms leading to NEBD are largely unidentified. Using RNA interference in *C. elegans* embryos and antibody inhibition experiments in *Xenopus* egg extracts we show that the nucleoporin gp210 is required for efficient NEBD in both systems. We also confirmed our previous results suggesting that gp210 is not required for nuclear membrane fusion and nuclear pore complex formation at the end of mitosis both in *C. elegans* and *Xenopus* egg extracts (Galy *et al.* (2003), Antonin *et al*. (2005)). This study unexpectedly suggests that gp210 functions in the context of NEBD and indicates that nuclear pore complex disassembly and NEBD are functionally linked.

43

### Lamin A/C Mutation Activates MAP Kinase Signaling Pathways in Hearts of an Animal Model of Autosomal Dominant Emery-Dreifuss Muscular Dystrophy

A. Muchir,[1] P. Pavlidis,[2] A. J. Herron,[3] T. Arimura,[4] G. Bonne,[5] H. J. Worman[1]; [1]Departments of Medicine and of Anatomy and Cell Biology,

Columbia University College of Physicians & Surgeons, New York, NY, [2]Department of Biomedical Informatics, Columbia University College of Physicians & Surgeons, New York, NY, [3]Institute of Comparative Medicine and Department of Pathology, Columbia University College of Physicians & Surgeons, New York, NY, [4]INSERM U582, Institut de Myologie, Groupe Hospitalier Pitie-Salpetriere, Paris, France, [5]INSERM U582, Institut de Myologie, Groupe Hospitalier Petie-Salpetriere, Paris, France

Mutations in LMNA gene encoding lamins A and C, two intermediate filaments of the nuclear envelope, cause diseases affecting various tissues, including striated muscle and the aging process. We have created a lamin A/C H222P mutant knock-in mouse model of Emery-Dreifuss muscular dystrophy, which develops skeletal muscle disease and cardiomyopathy. In the present study, we determined the effects of this mutation on signaling pathways that may be involved in the development of cardiac abnormalities. We analyzed genome-wide gene expression profiles in hearts from mutant and control mice using Affymetrix GeneChip® Mouse Genome 430 2.0 Array. To do not dilute our analysis with genes that are common to other cardiomyopathies, hearts were analyzed at an early stage before the appearance of clinical symptoms or gross pathological abnormalities. To analyze the data, we applied statistical methods that included ANOVA (P-value <0.05) and a false discovery rate analysis adjusted to a stringent 5% level. Statistically significant abnormal expression of genes related to the stress activated MAP kinases molecular pathway were present in both H222P+/- and H222P-/- hearts and confirmed by real-time PCR. Using immunoblot analysis, we then showed increases in ERK1/2, JNK1/2 and p38, MAP kinases that have been previously implicated in cardiac hypertrophy and heart failure. We also showed an increased content of several sarcomere proteins (actin, myosin, troponin T), angiogenesis proteins (VEGF-C, apelin) and atrial natriuretic factor, which are commonly affected in cardiomyopathies. These results identify for the first time a mechanism whereby lamin A/C mutation results in enhanced expression of MAP kinases, which could be a cornerstone in the development of cardiomyopathy in Emery-Dreifuss muscular dystrophy and related myopathies caused by LMNA mutations.

44

### Loss of the Dystonia-associated Protein TorsinA Selectively Disrupts the Neuronal Nuclear Envelope

R. E. Goodchild,[1] W. T. Dauer[2]; [1]Neurology, Columbia University, NYC, NY, [2]Neurology and Pharmacology, Columbia University, NYC, NY

A glutamic acid deletion (ΔE) in the ubiquitously expressed AAA+ protein torsinA causes the neurodevelopmental disease, DYT1 dystonia. Although the majority of torsinA resides within the ER, torsinA binds a substrate in the lumen of the nuclear envelope (NE), and the ΔE mutation enhances this interaction. Using a novel cell-based screen, we identified lamina-associated polypeptide 1 (LAP1) as a torsinA-interacting protein. LAP1 is required for the NE localization of torsinA and appears to interact with torsinA through a conserved luminal domain. In order to examine whether the NE function of torsinA is involved in the pathogenesis of DYT1 dystonia we developed torsinA null and ΔE 'knock-in' mice. Mice homozygous for the null or the ΔE mutation are phenocopies and both die shortly after birth. Neurons from these mice display severely disordered nuclear membranes that contain vesicles in the perinuclear space. These abnormalities are not present in non-neuronal cells. The presence and severity of nuclear membrane abnormalities shows a developmental dependence; they initially appear in post-migratory neurons and worsen concurrent with neuronal maturation. These observations imply that the neural specificity of DYT1 dystonia derives from a unique requirement of the neuronal nuclear envelope for torsinA function.

45

### Accumulation of S cerevisiae Esc1p causes Laminopathy-like Alterations of the Nuclear Envelope that are not Inherited by Daughter Nuclei

T. Hattier, E. Andrulis, A. M. Tartakoff; Pathology, Cell Biology Program and Molecular Biology, Case Western Reserve University, Cleveland, OH

The possible existence of a lamina which determines nuclear shape in yeast is controversial since lamin sequence homologs do not exist. Nevertheless, like lamins, S. cerevisiae Esc1p has coiled-coil domains and localizes to the nuclear periphery where it anchors heterochromatin. We observe that increased expression of Esc1p causes dramatic elaborations of the nuclear envelope (NE), which extend into the cytoplasm and are reminiscent of modifications of nuclear shape seen in human laminopathies. These double membrane extensions, here referred to as ESCapades, include nuclear pores, but only limited amounts of nucleoplasm. They often contact both the nucleolus and the vacuole. Time-lapse imaging shows that ESCapades normally originate at the nucleus-vacuole junction and often wrap around the vacuole. Strikingly, examination of cells in anaphase shows that ESCapades are not inherited by daughter nuclei. Contrary to expectation, exclusion of these structures from daughters is independent of septin function and the presence of vacuoles. Moreover, they are excluded even when nuclear division is restricted to the maternal cytoplasm. Thus, this behavior may reflect the existence of an intrinsically distinct "maternal domain" of the nuclear periphery. The exclusion of atypical structures provides a striking example of "phenotypic normalization" which may sustain continued cell growth when parental nuclei have acquired potentially deleterious characteristics. Consistent with this, retention of ESCapades significantly slows the pace of bud formation in mothers while the overall speed of growth is only modestly affected. Maternal retention of atypical NE structures may be unique to the closed mitoses of budding and fission yeast; higher eukaryotic cells appear able to cope with such issues by extensive disassembly and reassembly of the nuclear envelope during mitosis

## Regulation of the Cell Cycle (46-51)

46

### CaMKII Triggers Exit from Meiosis II by Sensitising the APC/C Inhibitor XErp1 for Degradation

N. Rauh, A. Schmidt, J. Bormann, E. A. Nigg, T. U. Mayer; Cell Biology, MPI of Biochemistry, Martinsried, Germany

Vertebrate eggs awaiting fertilisation are arrested at metaphase of meiosis II by a biochemical activity termed cytostatic factor (CSF). This activity inhibits the anaphase-promoting complex/cyclosome (APC/C), an ubiquitin-ligase that triggers anaphase onset and mitotic/meiotic exit by targeting securin and M-phase cyclins for destruction. Upon fertilisation a transient rise in free intracellular calcium causes release from CSF arrest and thus APC/C activation. While it is well established that CaMKII is the essential target of the calcium signal upon fertilisation, the relevant substrates of this kinase have not been identified and the mechanism(s) leading to APC/C activation have long remained obscure. Recently, we have identified XErp1 (Emi2), a novel component of CSF activity that is both necessary and sufficient to keep the APC/C inactive in CSF-arrested *Xenopus* egg extracts. Here we show that calcium-activated CaMKII triggers exit from meiosis II by sensitising the APC/C inhibitor XErp1 for Polo-like kinase

1 (Plx1)-dependent degradation. Phosphorylation of XErp1 by CaMKII leads to the recruitment of Plx1 which in turn triggers the destruction of XErp1 by phosphorylating a site known to serve as a phosphorylation-dependent degradation signal for the ubiquitin ligase $SCF^{\beta\text{-}TRCP}$. Our findings thus explain how a calcium signal prompts the exit from meiosis through the spatiotemporal integration of the action of two key kinases, CaMKII and Plx1, both converging onto XErp1, a critical inhibitor of APC/C.

47

**Bypassing the Positive-Feedback Loops in Cdc2 Activation Uncouples the Biochemical Oscillator from Mitotic Progression**

J. R. Pomerening, E. Petschnigg, J. E. Ferrell; Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA

Enzymatic control of the mitotic cell cycle ensures discrete phases of DNA replication, growth, and division. Progression into and exit from mitosis is regulated by oscillations in the activity of Cdc2-cyclin B kinase. The negative-feedback loop of this oscillator is driven by the anaphase-promoting complex (APC), which inactivates Cdc2 via cyclin B proteolysis, and controls the metaphase to anaphase transition, as well as mitotic exit. The upstrokes of Cdc2-cyclin B activity are powered by positive-feedback loops, and while positive feedback is known to elicit useful behaviors such as ultrasensitivity and bistability from cell-signaling modules, the biological importance of these characters in defining the systems-level behavior of the Cdc2/APC oscillator is not well understood. By applying a positive-feedback-insensitive mutant of human Cdc2 (Cdc2T14AY15F), Cdc2AF, we short-circuited the normal pathway of Cdc2 activation in HeLa cells. By using multiple cell cycle biosensors and live-cell imaging, we discovered that bypassing the positive-feedback loops involved in Cdc2 activation uncouples the biochemical oscillations in Cdc2 activity from the physiological events that must occur during mitosis. A lamin A-YFP biosensor revealed that nuclear envelope breakdown and nuclear envelope reformation events without cytokinesis could occur multiple times within the same Cdc2AF-transfected cell. A cyclin B1-YFP biosensor revealed that wild-type Cdc2-transfected cells would undergo one round of cyclin stability/proteolysis and division during a 12-hour period, whereas cells transfected with Cdc2AF would undergo multiple periods of cyclin stability/instability - up to six - within the same, non-dividing cells during a 12-hour period. Finally, using kinase-activity assays and multivariate flow cytometry, we confirmed that short-circuiting these positive-feedback loops causes somatic cells to undergo repetitive biochemical pseudo-cycles without being able to actually initiate and progress through mitosis.

48

**Aurora-A Kinase is Required for Timely Nuclear Envelope Breakdown in C elegans**

N. C. Portier, P. Maddox, K. Oegema; Ludwig Inst. for Cancer Research, La Jolla, CA

Aurora-A is a mitotic serine/threonine kinase that localizes to centrosomes, the major microtubule organizing centers in animal cells. The gene encoding Aurora-A is amplified in many cancers, possibly because overexpression overrides critical checkpoints, promoting genetic instability. It has been proposed that centrosomes influence cell cycle progression by focusing cell cycle regulators. Consistent with this idea, Aurora-A has been shown to regulate both the recruitment of pericentriolar material during mitotic entry (centrosome maturation), and G2/M progression. We used the C. elegans early embryo to investigate the relationship between the role of Aurora-A in centrosome maturation and cell cycle progression. Upon fertilization, the oocyte-derived nucleus undergoes two meiotic divisions. The resulting haploid pronuclei replicate their DNA, the chromatin condenses, and the nuclear envelopes break down (NEBD). We imaged embryos expressing fluorescently-tagged histones to visualize chromosomes and thus determine cell cycle milestones. We found that depletion of Aurora-A by RNA-mediated interference extends the time interval between anaphase of meiosis II and NEBD in mitosis. By using a quantitative light-microscopy-based live assay to measure chromosome condensation we showed that depletion of aurora-A increases the interval between the onset of chromosome condensation and NEBD. However, the rate of chromosome condensation is unaffected in depleted embryos. This result suggests that the timing of mitotic entry is not affected by Aurora-A depletion, but that NEBD is specifically delayed. Disruption of microtubule-nuclear envelope interactions by nocodazole treatment or dynein depletion did not affect the timing of NEBD, indicating that centrosomes have a role in NEBD that is independent of microtubules. Depletion of other proteins required for centrosome assembly delays NEBD, but to a lesser extent than depletion of Aurora-A. This suggests a centrosome-independent contribution of Aurora-A to NEBD.

49

**Mto1p and Mto2p Regulate the Function of the Fission Yeast γ-TuC at the MTOCs**

S. Venkatram, K. L. Gould; Cell and Developmental Biology, Vanderbilt University Medical School, Nashville, TN

Microtubules (MTs) in fission yeast are organized by three different microtubule-organizing centers (MTOC): the interphase MTOCs (iMTOC) that organize cytoplasmic microtubules, the spindle pole bodies (SPBs) that organize the mitotic spindle and astral MTs and the equatorial MTOC (eMTOC) that organizes the post-anaphase array of MTs. Using proteomic approaches, we identified Mto1p and Mto2p, which associate with the gamma-tubulin complex (γ-TuC), and are important for proper organization of all cytoplasmic MTs. Consistent with its role in MT organization, *mto1*Δ cells exhibit defects in cell polarity, nuclear positioning, astral MT formation and cleavage plane specification. In addition, we have shown that the eMTOC is critical for proper anchoring of the cytokinetic actin ring during cytokinesis. How does the g-TuC, specifically Mto1p and Mto2p perform these functions? In this study, we have identified Myp2p, a non-essential myosin II component of the actin ring, as a possible link between the ring and the eMTOC and hence perform a role in ring anchoring by the eMTOC. Moreover, structure/function analyses of Mto1p and Mto2p have revealed that distinct domains of these proteins target them to the different MTOCs and contribute to different aspects of their function in association with the γ-TuC. Significantly, we have found that Mto1p is a microtubule-binding protein suggesting that Mto1p links the γ-TuC to cytoplasmic MTs. Also, both Mto1p and Mto2p are phosphoproteins and their phosphorylation status is regulated with respect to the cell cycle. The role of this phosphorylation in Mto2p function will also be presented. Taken together, our studies suggest a model in which distinct domains of Mto1p and Mto2p contribute to their function in organizing different MT structures and help anchor the cytokinetic actin ring through interaction with conserved ring components.

50

**Cellprofiler: Free Software for Automatically Measuring Cells in Images**

C. M. Clarke,[1] A. E. Carpenter,[2] T. R. Jones,[1] D. B. Wheeler,[2] O. Friman,[3] J. Chang,[2] D. A. Guertin,[2] I. Kang,[1] P. Golland,[1] D. M. Sabaini[2]; [1]MIT,

Cambridge, MA, [2]Whitehead, Cambridge, MA, [3]Harvard, Cambridge, MA
Advances in imaging hardware now allow the rapid collection of thousands of high resolution images of cells. Automatically measuring features of cells quantitatively from these images has been difficult due to the limitations and often proprietary nature of available image analysis software. We have therefore developed CellProfiler cell image analysis software to allow biologists without training in computer vision or programming to quantitatively measure cells in thousands of images automatically, without tedious user interacion. This freely available, open-source software project is modular, allowing simple adaptation to a variety of cell types and phenotypes. CellProfiler is compatible with common image formats (bmp, cur, dib, fts, fits, gif, hdf, ico, jpg, jpeg, pbm, pcx, pgm, png, pnm, ppm, ras, tif, tiff, xwd) and movie formats (avi, stk). Here we describe and validate the software using cells from human, mouse, yeast, and fruit fly to measure phenotypes including cell count, cell size, cell cycle distribution, and the levels and localization of proteins and phospho-proteins, including application to time-lapse and high-throughput experiments.

51

**Requirement For Cdk4 Kinase Function In Breast Cancer**

P. Sicinski; Harvard Medical School, Boston, MA
Cyclin D1 protein is overexpressed in the majority of human breast cancers. We previously found that mice lacking cyclin D1 are resistant to mammary carcinomas trigged by ErbB-2 oncogene. In the current study we investigated which function of cyclin D1 is required for ErbB-2-driven mammary oncogenesis. We report that the ability of cyclin D1 to activate cyclin-dependent kinase CDK4 underlies the critical role for cyclin D1 in breast cancer formation. We also found that the continued presence of CDK4-associated kinase activity is critically required for breast tumorigenesis. We analyzed primary human breast cancers and found high cyclin D1 levels in a subset (approx. 25%) of tumors with ErbB-2 amplification. These D1-high, ErbB-2-overexpressing breast cancers have particularly poor prognosis. We propose that this subset of breast cancer patients might benefit from inhibiting CDK4 kinase activity.

## Signaling in the Immune System (52-57)

52

**T Cell Receptor Nanoclusters Sustain Signaling in the Immunological Synapse**

M. L. Dustin, R. Varma, G. Campi, T. N. Sims; Pathology, NYU SOM, New York, NY
Dynamic TCR-MHC-peptide (MHCp) interactions sustain T cell signaling. The site of these dynamic events in the stable immunological synapses is unknown. We have utilized primary T cells from T cell receptor (TCR) transgenic mice interacting with supported planar bilayers containing MHCp and ICAM-1. The interface between the T cell and planar bilayers is defined as the synapse. We have found that TCR-MHCp engagement occurs continuously in the periphery of the synapse leading to clustering, phosphorylation and translocation of the TCRs towards the center where TCR-MHCp interactions accumulate. Perturbations that stop TCR signaling, such as anti-MHCp antibodies or disruption of actin cytoskeleton, also stop formation and translocation of TCR nanoclusters, but have no immediate impact on the central TCR macrocluster. The non-receptor tyrosine kinase ZAP-70 is recruited to the TCR nanoclusters, which are also sites of tyrosine phosphorylation. Thus, TCR engagement required for signaling is continuously renewed in the periphery of the immunological synapse. We suggest the immunological synapse has at least three distinct signaling zones, a peripheral lamellipodium where signaling is initiated, an intermediate lamella where signaling is amplified and sustained and a central zone where microclusters converge and tyrosine kinase signaling is extinguished.

53

**Single Molecule Imaging of Immunological Synapse Formation in Planar Bilayer-Stimulated T Cells**

A. D. Douglass, R. D. Vale; HHMI and Department of Cell and Molecular Pharmacology, UC San Francisco, San Francisco, CA
We have developed a method of stimulating immortalized, Jurkat T cells with supported lipid bilayers that contain adhesion molecules (CD58 or ICAM-1) as well as antibodies directed against the T cell receptor (TCR). In addition, we can follow the dynamic behavior of other signaling and adhesion proteins with GFP tags. The anti-TCR antibodies cause the TCR to coalesce into microclusters at the cell periphery, and these microclusters are then transported to the center of the contact by retrograde actin flow. The central region of TCR accumulation (termed the “cSMAC”) is surrounded by a ring of LFA-1 (the “pSMAC”), an arrangement that is reminiscent of the immunological synapse that forms at the interface between a T cell and an antigen-presenting cell. Surprisingly, several proteins involved in transducing signals from the TCR, such as the adaptor protein, LAT, and the kinase, Lck, preferentially localize to regions of the cell-bilayer interface that are enriched in the coreceptor CD2, rather than to TCR-rich clusters. Through single molecule imaging by total internal reflection fluorescence (TIRF), we are in the process of creating a “diffusion map” of the Jurkat immunological synapse. Our preliminary work shows boundaries between the lamella, pSMAC and cSMAC that often appear to constitute diffusion barriers that limit the movement of proteins between compartments. Thus, our work shows that Jurkat cells will spatially organize molecules in a manner than resembles the immunological synapse of native T cells. We also show the rapid formation of signaling microclusters in the lamella, the actin-based transport of these clusters towards the cell center to generate the cSMAC, and we provide the first detailed analysis of diffusion dynamics within the immunological synapse.

54

**Automated Real-Time Image Cross-Correlation Analysis of Mast Cell Signaling Reveals Detailed Dynamics of Early Signaling Events**

R. Das, D. Holowka, B. Baird; Chemistry and Chemical Biology, Cornell University, Ithaca, NY
Signaling in mast cells and basophils is mediated through IgE and its high affinity cell surface receptor Fc-epsilon-RI. Crosslinking of the receptors by a cognate multivalent antigen initiates a signal cascade leading to degranulation and release of mediators of the allergic immune response. We probe the spatio-temporal dynamics of fluorescent chimeras of signaling proteins involved in the early steps of this signal cascade using real-time multicolor confocal fluorescence microscopy. We image living RBL-2H3 mast cells at physiologically relevant temperatures following stimulation with multivalent antigen and acquire sequential images with a 3s time resolution. A fluorescent tag on the antigen allows us to visualize the plasma membrane localization of crosslinked receptors. We have developed an automated image analysis scheme that allows us to rapidly quantify the recruitment of fluorescent intracellular proteins to the plasma membrane and their colocalization with crosslinked receptors as measured by a cross

correlation between the plasma membrane distribution of the two fluorophores. We systematically apply this analysis to characterize stimulated interactions of several chimeric proteins, including the tyrosine kinases Lyn and Syk, and the adaptor protein LAT. Using an Akt PH domain chimera, we find evidence for a time-dependent increase in localized phosphatidylinositol-(3,4,5)-trisphosphate ($PIP_3$) production at sites of receptor aggregation.

55

**High Affinity LFA-1 is Recruited to The Leading Edge During T Cell Migration and Immunological Synapse Formation**

N. A. Morin,[1] M. Shimaoka,[2] T. A. Springer,[2] J. W. Janowski,[1] J. S. Reichner,[1] M. Kim[1]; [1]Surgery, Rhode Island Hospital, Brown Medical School, Providence, RI, [2]The CBR Institute for Biomedical Research, Inc., Boston, MA

Cell migration is central to many biological and pathological processes, including embryogenesis, tissue repair and regeneration as well as cancer and the inflammatory response. Leukocyte migration from blood into lymphoid tissues or sites of inflammation occurs through interactions of cell surface integrins with their ligands expressed on the vascular endothelium and the extracellular matrix. Integrin LFA-1 (αLβ2) is a critical component in the effective trafficking of leukocytes. We hypothesize that only a small subpopulation of LFA-1 on the surface of T cells is activated by chemokine stimulation and the high affinity LFA-1 is localized at the leading edge of the polarized cells in a spatially restricted manner during migration. Quantitative analysis of LFA-1 activation with activation specific monoclonal antibodies showed only a small population of total LFA-1 on human T cell is activated by stimulation with SDF-1. Confocal microscopy and 3D reconstruction images of LFA-1 immunofluororescence staining with conformation specific (active vs inactive) monoclonal antibodies revealed the distinct three-dimensional distribution of the active conformations of LFA-1 at the leading edge of T cells engaged in migration and the cell-cell interface during immunological synapse formation. Selective inhibition of the active form of LFA-1 was sufficient to block T cell transendothelial migration. Dynamic fluorescence resonance energy transfer (FRET) measurements of Rap-1 CFP/YFP FRET sensor on live migrating primary T cell showed the distribution of Rap-1 activation is mainly localized near the nucleus and periodic strong Rap-1 activation spread toward the membrane protrusion. These data suggest that only a small subpopulation of LFA-1 at the leading edge of polarized T cells is functionally activated and competent to mediate migration and immunological synapse formation.

56

**The Alpha4 Integrin-Paxillin Interaction in Lymphocyte Trafficking during Development and Inflammation**

D. M. Rose,[1] C. C. Feral,[2] A. D. Sydlaske,[1] P. Scott,[1] M. H. Ginsberg[2]; [1]Medicine, VA Medical Center/UCSD, San Diego, CA, [2]Medicine, UCSD, San Diego, CA

VLA-4 integrins (α4β1) function in lymphocyte trafficking through compartments of the immune system during immune surveillance and inflammation. We have previously described the direct physical interaction of the α4 integrin cytoplasmic domain with the signaling adapter molecule, paxillin, and defined the importance of this interaction in lymphocyte migration in vitro. To determine the role of the α4-paxillin interaction in lymphocyte trafficking during the development of the immune system and inflammation, a novel mouse strain was generated with a selective mutation (Y991A) introduced into the α4 gene that disrupts paxillin binding to the α4 cytoplasmic domain. Mice homozygous for the mutation were viable with no gross abnormalities in development. The α4Y991A mice had no defect in hematopoiesis as judged by abundance of different cell populations in the blood. Furthermore, the architecture and lymphocyte subpopulations in primary and secondary lymphoid tissues of α4Y991A mice were similar to α4WT mice. However, upon challenge, α4Y991A mice had a significant defect in lymphocyte recruitment during thioglycollate-induced peritonitis. This was selective for lymphocytes as no difference was observed in neutrophil recruitment to the peritonea between α4Y991A and α4WT mice. To further study lymphocyte trafficking during inflammatory and non-inflammatory conditions, splenocytes were isolated from α4WT and α4Y991A mice and differentially labeled. Mixed cell populations were introduced back into α4WT mice by tail vein injection, and trafficking of labeled splenocytes to spleens and peritonea of thioglycollate-treated mice was followed by flow cytometry. While α4WT and α4Y991A cells migrated equally well to the spleen, there was a marked reduction in migration of α4Y991A splenocytes to the inflamed peritoneum. These results indicate that the α4-paxillin interaction plays a selective role in lymphocyte trafficking to sites of inflammation, but not in trafficking during development and homeostasis.

57

**The Differential Roles of Ezrin and Moesin in Immune Synapse Formation**

T. Ilani, A. Bretscher; MB&G, Cornell University, Ithaca, NY

The immune synapse (IS) is the specialized junction between T-cell and antigen presenting cell (APC) characterized by clustering of T-cell receptors and their associated proteins. IS forms within seconds following T cell-APC recognition and persists for hours. The cytoskeleton plays a major role in this molecular reorganization, so an understanding of the regulated attachment of membrane proteins to the cytoskeleton lies at the heart of IS formation. The ERM (Ezrin/Radixin/Moesin) proteins, which provide a regulated linkage between membrane proteins and the actin cytoskeleton, have been implicated in T-cell activation and IS formation. The activity of ERM proteins is regulated by reversible phosphorylation at a conserved residue, which opens the molecule allowing its linking functions. The existence of three ERM members is frequently referred to as redundancy. Nonetheless, using confocal microscopy and biochemical methods we found that in both a T-cell line and freshly isolated human T-cells ezrin and moesin are differentially distributed. In resting T-cells moesin is restricted to the cell membrane and is in its active phosphorylated form, whereas ezrin shows a cytoplasmic distribution and is in its dormant non-phosphorylated form. Following T-cell receptor activation ezrin relocalizes to the membrane where it is subsequently phosphorylated while moesin stays localized at the membrane and shows a rapid and transient dephosphorylation. We further analyzed the time course of spatial changes and activity regulation of ERM during IS formation. Upon T-cell receptor engagement ezrin is localized at the activation site along with CD3 and PKC-θ whereas moesin is rapidly dephosphorylated and removed from the activation sitetogether with CD43. These distinctions are consistent throughout synapse maturation and point to a differential role for these proteins in IS formation.

## EB Wilson Medal Presentation & Lecture (58)

58

### SnRNPs: Cellular and Viral Regulators of Gene Expression

J. A. Steitz; Dept MBB/HHMI, Yale University, New Haven, CT

Small nuclear RNAs (snRNAs), the most famous of which function in the removal of introns from pre-mRNAs, are important contributors to gene expression. The discovery of snRNAs complexed with proteins to form small nuclear ribonucleoproteins (snRNPs) will first be reviewed. A second class of snRNPs guides the introduction of nucleotide modifications such as pseudouridine and 2'-O-methyl groups into other RNAs. Insight into the function of pseudouridine modifications has recently come from an unexpected source - study of the U7 snRNP, which directs the 3'-end maturation of metazoan histone mRNAs. Nucleotide analog interference mapping (NAIM) and conventional mutagenesis reveal that the ability of a particular sugar moiety within the Sm site to assume the 2'-endo conformation is essential for U7 snRNP assembly. Pseudouridine interferes with Sm protein binding by rigidifying the backbone in the 3'-endo conformation. This argues that conserved pseudouridine modifications in rRNA and snRNAs are introduced where it is functionally critical to fix the backbone in the 3'-endo conformation. Viruses often provide powerful tools to dissect gene regulation. Kaposi's sarcoma-associated herpesvirus (KSHV) produces a 1 kb non-coding, polyadenylated, nuclear RNA called PAN in lytically infected human cells. PAN contains a novel post-transcriptional element, the PAN-ENE (PAN expression and nuclear retention element), essential for its high levels of accumulation. Insertion of the PAN-ENE is also sufficient to significantly increase RNA expression from an otherwise inefficiently expressed intronless b-globin construct, but does not concomitantly enhance the production of encoded protein; rather, the unspliced b-globin mRNA is retained in the nucleus. The nuclear retention activity of the PAN-ENE can be overcome by tethering export factors or by inserting an intron into the mRNA. Recent advances provide insights into the mechanism of action of the PAN-ENE in regulating the levels of nuclear RNAs.

## Growth Factors & Receptors (59-82)

59

### Control of EGF Receptor Endocytosis by Receptor Dimerization, Rather Than Receptor Kinase Activation

Q. Wang,[1] G. Villeneuve,[2] Z. Wang[1]; [1]Department of Cell Biology, University of Alberta, Edmonton, AB, Canada, [2]University of Alberta, Edmonton, AB, Canada

Given that ligand binding is essential for the rapid internalization of epidermal growth factor (EGF) receptor (EGFR), the events induced by the ligand binding likely contribute to the regulation of EGFR internalization. These events include receptor dimerization, activation of intrinsic tyrosine kinase activity and autophosphorylation. While the initial results are very controversial regarding the role of EGFR kinase activity in EGFR internalization, more recent data suggest that EGFR kinase activation is essential for EGFR internalization. However, we showed here that inhibition of EGFR kinase activation by mutation or chemical inhibitors did not block EGF-induced EGFR internalization. Instead, EGFR dimerization is necessary and sufficient to stimulate EGFR internalization. We conclude that EGFR internalization is controlled by EGFR dimerization, rather than EGFR kinase activation. Our results also define a new role for EGFR dimerization: EGFR dimerization by itself can drive EGFR internalization, independent of its role in the activation of EGFR kinase.

60

### Receptor Downregulation Confers Response Robustness upon the EGFR System

H. Shankaran, H. S. Wiley, H. Resat; Pacific Northwest National Laboratory, Richland, WA

OBJECTIVE: The epidermal growth factor receptor (EGFR) plays a critical role in proliferation and differentiation. An important feature of EGFR signaling is that receptor internalization is enhanced when EGFR binds its ligand EGF. This property termed downregulation is viewed as a mechanism for signal termination. Here, we employ mathematical modeling to explore the role of downregulation in controlling the input-output characteristics of this signaling system. METHODS: We constructed a mathematical model that includes EGF-EGFR binding, EGFR synthesis and internalization. We quantified the extent of downregulation $\delta$ as the ratio of the internalization rate of receptor-ligand complexes to that of free receptors. We ran the model to determine the evolution of surface receptor-ligand complexes (system output) in response to time-varying sinusoidal ligand profiles (system input) over a range of $\delta$ values. RESULTS: The response delay is the time-difference between the peaks of the output and input profiles, and can be used as a performance measure. For $\delta$ >10, the system output matches the input remarkably well with a delay of ~10 min irrespective of the input wavelength, $\lambda$ in the range $\lambda$=100-1000 min. The EGF-EGFR system has a $\delta$ of ~20-30, and falls in this robust region of the parameter space. Reducing $\delta$ increases response delay in addition to making it a function of the input wavelength. For the case of no downregulation ($\delta$=1), the delay varies from 25 min for $\lambda$=100 min to 110 min for $\lambda$=1000 min. CONCLUSIONS: Downregulation enables the EGF-EGFR system to generate outputs that robustly reproduce the input profile over a wide-range of wavelengths. We believe that investigating elements of biochemical networks in this fashion to elucidate their role in controlling input-output characteristics will prove to be invaluable in constructing and understanding complex whole cell models.

61

### PKA, but not Epac, Increases the Ligand-dependent Activation of ErbB/HER Receptors and Proliferation of Cultured Schwann Cells

P. V. Monje, M. Bartlett Bunge, P. M. Wood; University of Miami School of Medicine, Miami Project to Cure Paralysis, Miami, FL

The elevation of intracellular cAMP synergistically enhances the heregulin-dependent proliferation of cultured Schwann cells (SCs); however, the mechanism by which this occurs has not been completely defined. To better understand this mechanism, we studied the effect of pharmacological inhibitors of protein kinase A (PKA) and cell permeable cAMP analogs with demonstrated specificity for activation of PKA, or Epac (exchange protein activated by cAMP), on heregulin-induced proliferation. We observed that activation of PKA, but not Epac, synergistically increased heregulin-dependent proliferation of both adult human and rat SCs. We further observed that cAMP signaling through PKA synergistically increased the ligand-dependent activation of the neuregulin receptors ErbB2/3 (HER2/3) and downstream signaling in the mitogen-activated protein kinase (MAPK) and the phosphatidyl-inositol-3-kinase (PI3-K)-Akt pathways. The activation of PKA by forskolin or by cAMP analogs did

not result in receptor phosphorylation when administered in the absence of heregulin. Consistent with these observations, physiological changes in cAMP promoted by the stimulation of several G-protein coupled receptors, including adenosine and adrenaline receptors, synergistically enhanced ErbB/HER phosphorylation and SC proliferation in response to heregulin. Overall, our results suggest a novel mechanism in which agents that elevate cAMP increase the ligand-induced activation of heregulin receptors by a PKA-dependent pathway and thereby increase cell cycle progression in SCs.

62

**Caveolin-1 Phosphorylation Under Oxidative Stress is Required for Caveolae-mediated Perinuclear Trafficking of the EGF Receptor**

E. M. Khan, J. M. Heidinger, M. Levy, T. Ravid, T. Goldkorn; Internal Medicine - Pulmonary, University of California, Davis, Davis, CA

The epidermal growth factor (EGF) receptor (EGFR) is over-expressed in several types of cancer cells and the regulation of its oncogenic potential has been widely studied. The inability of the EGFR to be down-regulated via clathrin-mediated endocytosis and degradation has been linked to its oncogenicity. The paradigm for EGFR down-regulation involves the trafficking of activated receptor molecules from the plasma membrane, through clathrin-coated pits, and into the cell for lysosomal degradation. We have previously shown that oxidative stress generated by $H_2O_2$ results in aberrant phosphorylation of the EGFR. This results in the loss of c-Cbl-mediated ubiquitination of the EGFR and, consequently, prevents its degradation. However, we have found that c-Cbl-mediated ubiquitination is required solely for degradation but not for internalization of EGFR under oxidative stress. To further examine the fate of the EGFR under oxidative stress, we used confocal analysis to show that the receptor not only remains co-localized with caveolin-1 at the plasma membrane, but at longer time points, is also sorted to a perinuclear compartment via a clathrin-independent, caveolae-mediated pathway. Our findings indicate that although EGFR associates with caveolin-1 constitutively, caveolin-1 is hyper-phosphorylated only under oxidative stress, which is essential in transporting EGFR to a perinuclear location, where it is not degraded and remains active. Hence, oxidative stress may have a role in tumorigenesis by not only activating the EGFR, but also by promoting prolonged activation of the receptor both at the plasma membrane and within the cell.

63

**The Leucine-Rich Repeat Protein LRIG1 Inhibits ErbB Mediated Breast Tumor Cell Growth**

J. K. Miller, D. L. Shattuck, M. B. Laederich, K. L. Carraway III, C. A. Sweeney; Biochemistry and Molecular Biology, University of California, Davis, Sacramento, CA

Receptor tyrosine kinases (RTKs) are key mediators of normal cellular processes such as the development and maintenance of tissues, and are also critically involved in the growth and progression of human cancers. One subfamily of RTKs is the epidermal growth factor (EGF) family which consists of four members: EGF receptor (EGFR), ErbB2, ErbB3, and ErbB4. Binding of EGF-like growth factors induces a global signaling cascade that affects varied downstream processes including proliferation, motility, and survival. Aberrant activation of this family of receptors is strongly linked to the generation and progression of breast cancer. While small molecule inhibitors and monoclonal antibodies have had some therapeutic success, endogenous proteins may also be exploited to downregulate receptor levels in tumor cells. Specifically, the leucine-rich repeat protein, LRIG1, has been previously shown by our lab to negatively regulate all four members of the ErbB family, and LRIG1-mediated receptor ubiquitination and degradation may contribute to the suppression of ErbB receptor function. Here we seek to determine whether LRIG1 can regulate endogenous receptors in cultured breast tumor cell lines. Through the use of viral mediated transduction, we have found that endogenous ErbB receptors are reduced following transduction with LRIG1. In addition, these transduced cell lines display a concomitant decrease in signaling and, ultimately, proliferation in response to EGF-like growth factors. These data suggest that increasing levels of LRIG1 can counteract aberrant ErbB signaling and decrease proliferation in breast lines responsive to EGF-like growth factors. Furthermore, the LRIG1 protein could hold either therapeutic or prognostic value for breast cancer treatments.

64

**Regulated Shedding of EGFR Ligands is Controlled by Oligomerization**

G. J. Newton, L. K. Opresko, D. J. Panther, G. Orr, R. D. Quesenberry, H. S. Wiley; Biological Sciences, Pacific Northwest National Laboratory, Richland, WA

The epidermal growth factor receptor (EGFR) can be activated by seven ligands that are initially synthesized as membrane-bound precursors. We have found that these ligands can be classified as being either slowly or rapidly released, based on their shedding rates measured using quantitative assays. To investigate the structural basis for these differences, we constructed chimeras of TGFα (high release) and HB-EGF (low release) in which we swapped the stem, transmembrane (TM), and cytoplasmic domains between the two ligands. After expressing the chimeric ligands in Chinese hamster ovary cells, we measured the ligand release rate relative to the total level of expressed ligand (fractional release). We found that replacing either the stem or TM domain of HB-EGF with those from TGFα significantly increased its rate of release, suggesting that some structural aspect of the ligand inhibited shedding. To test our hypothesis that these regions are involved in ligand oligomerization, we used site-directed mutagenesis to change three charged polar residues in the stem and three uncharged polar residues in the TM domain to either alanine or valine. We observed a significant increase in shedding following mutations in either the stem or TM domains, but double mutations displayed shedding rates similar to TGFα. We used fluorescent resonance energy transfer (FRET) to directly demonstrate oligomerization of the chimeras and observed an inverse relationship between the degree of FRET and the fractional release of the ligands. In the double mutant that was rapidly shed, there was no detectable FRET, indicating that these ligands do not form oligomers. Taken together, our results suggest that EGFR ligands can form oligomers mediated by intermolecular interactions within both the stem and TM domains. In turn, the degree of ligand oligomerization can control the regulated shedding rate.

65

**The Carboxy-terminal Domain of HB-EGF Regulates Bcl6, a Transcriptional Repressor, via Direct Interaction**

Y. Kinugasa, M. Hieda, S. Higashiyama; School of Medicine, Ehime University, Ehime, Japan

Heparin-binding EGF-like growth factor, a member of the EGF family is synthesized as a type I transmenbrane precursor (proHB-EGF) containing of the extracellular EGF-like domain and a cytoplasmic tail. Proteolytic cleavage of extracellular domain yields the soluble EGF-like growth factor

(sHB-EGF) and the transmembrane-cytoplasmic fragment (HB-EGF-TMC). Recently we demonstrated that transmembrane remnant, HB-EGF-TMC, translocates from the plasma membrane to the nucleus. Its nuclear translocation triggered nuclear export of a transcriptional repressor, PLZF and then the transcriptional suppression by PLZF is abolished. To establish the general nature of the transcriptional regulation by HB-EGF-TMC, we attempted to find out other transcriptional repressors regulated by HB-EGF-TMC. PLZF contains an amino-terminal BTB domain and carboxyl-terminal Krüppel-type zinc finger motifs, which is essential for the complex formation with HB-EGF-TMC. We screened transcriptional repressors having BTB domain and zinc finger motifs and found that a transcriptional repressor, B cell lymphoma 6 (Bcl6) interacts with HB-EGF-TMC. Transient transfection and co-precipitation experiment showed the interaction between Bcl6 and HB-EGF-TMC in vivo. Recombinant Bcl6 directly bound to cytoplasmic domain of HB-EGF via its zinc finger motifs in AlphaScreen assay. Bcl6 represses the cyclinD2 gene transcription. HB-EGF-TMC signaling abolished the transcriptional repression by Bcl6 in luciferase reporter assay. Moreover HB-EGF-TMC signaling activated the cyclinD2 protein expression in several lines of cultured cells. These findings show a novel transcriptional regulation; after releasing EGF-like growth factor, HB-EGF-TMC translocates to the nucleus and activates the transcription. As a result proHB-EGF is bidirectional signaling molecules, soluble EGF-like growth factor activates receptor tyrosine kinase in one direction and membrane-tethered fragment, HB-EGF-TMC regulates transcription in the other direction. We will also discuss the detailed localization of HB-EGF-TMC in the nucleus and its role in the transcriptional regulation

66

**P2Y1 Nucleotide-Receptors Increase Cell Proliferation Trans-Activating the EGF Receptor**

S. Buvinic, M. Bravo-Zehnder, J. Huidobro-Toro, A. González; Depto. Reumatología, Fac. Medicina, and Centro de Regulación Celular y Patología, Fac. Ciencias Biológicas. P. Universidad Católica de Chile, and MIFAB, Santiago, Chile

Metabotropic P2Y1 receptors (P2Y1R) are activated by extracellular nucleotides and regulate a variety of physiological events. Most studies on P2Y1R focused their role in rapid processes that depend on a rise in intracellular calcium, remaining unknown whether they exert long-term effects. Several G protein-coupled receptors (GPCR) modulate cell proliferation trans-activating the epidermal growth factor receptor (EGFR). Nucleotides are continuously released from cells and therefore they might act as local trophic factors. We found that epithelial FRT cells and HeLa cells responded to the selective P2Y1R agonist 2-MeSADP by increasing [H3]-thymidine incorporation with EC50 values of 42 ± 7 and 76 ± 3 nM, respectively; MRS 2179, a P2Y1R antagonist, abolished this effect. Interestingly, 2-MeSADP also increased the tyrosine phosphorylation of EGFR, by 3-4 fold in FRT cells and 60 fold in HeLa cells, leading to ERK1/2 activation. Inhibitors of EGFR kinase (AG1478), PKC (Ro318220) and metalloproteases (Ilomastat) reduced proliferation induced by 2-MeSADP. Strikingly, degradation of endogenously released nucleotides with apyrase reduced by 15-25% the basal proliferation rate of FRT and HeLa cells. Furthermore, epithelial MDCK cells overexpressing P2Y1R increased their proliferation rate through a mechanism that depended on EGFR activation. These results revealed that P2Y1R stimulation trans-activates EGFR leading to increased proliferation of both epithelial cells and cancerous cells, thus contributing to maintain the basal proliferation rate of these cells. Because nucleotides are widely released after cell damage and tissue injury, they could also play a role in tissue repair. (Funded by FONDAP grant 13980001 and Millennium project from MIDEPLAN).

67

**Conditional Activation of FGFR1 and FGFR2 in HC11 Mouse Mammary Epithelial 3D Cultures Differentially Regulates Cell Survival and Invasion**

W. Xian, K. L. Schwertfeger, T. C. Vargo-Gogola, J. M. Rosen; Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX

Fibroblast growth factors and their respective receptors (FGFR) have been implicated in mediating various aspects of mammary gland development and cancer progression. Although FGFR1 and FGFR2 share considerable amino acid sequence homology, these two receptor tyrosine kinases (RTKs) exhibit unique expression patterns and ligand binding capacities, exerting distinct effects during development and cancer progression. To date, most studies of FGFR signaling have been performed in 3T3 fibroblasts and other non-epithelial cells in two-dimensional culture systems and have not differentiated between effects of specific ligands. Thus, the unique signaling pathways and phenotypes elicited by these two highly related RTKs in polarized epitheli*a* have not been examined. Accordingly, we have developed an *in vitro* three-dimensional HC11 mouse mammary epithelial cell culture model combined with a ligand-independent, chemically-inducible FGFR dimerization system. Using this model we have observed distinct phenotypic and signaling differences following activation of iFGFR1 or iFGFR2. iFGFR1 activation resulted in high-level and sustained ERK phosphorylation, as well as differential effects on FRS2 phosphorylation as compared to iFGFR2. While activation of both RTKs in growth-arrested and polarized mammary acini led to reinitiation of cell proliferation and loss of cell polarity, only iFGFR1 activation increased cell survival. In contrast, iFGFR2 activation resulted in dramatically increased cell death even in the outer layer of cells in direct contact with the basal lamina. This appears to be a consequence of rapid tyrosine dephosphorylation of focal adhesion kinase following iFGFR2 activation. Additionally, dimerization of iFGFR1, but not iFGFR2, caused increased HC11 cell invasive properties and EMT. These results indicate that FGFR1 and FGFR2 may contribute differently to the etiology and progression of breast cancer. Supported in part by NIH grant CA16303.

68

**Endogenous FGF2 is Necessary for the Mitogenic Response to Prostaglandin F2a**

M. Sabbieti,[1] D. Agas,[1] L. Xiao,[1] L. Marchetti,[2] M. M. Hurley[1]; [1]Endocrinology and Metabolism, University of Connecticut Health Center, Farmington, CT, [2]Comparative Morphology and Biochemistry, University of Camerino, Camerino, Italy

Previous studies demonstrated that PGF2a regulated FGF2 and FGF receptor 2 mRNAs expression in Py1a osteoblastic cells and induced nuclear accumulation and co-localization of FGF2/FGFR2 by both protein kinase C and p44/42 ERK mechanisms. To study the biological relevance of PGF2a induced FGF-2/FGFR2 nuclear localization we used, as a model, primary calvarial osteoblasts from neonatal Fgf2+/+ and Fgf2-/- mice. Dose response studies showed that 24 h treatment with PGF2a (10-5 and 10-6M) significantly increased proliferation only in Fgf2+/+ osteoblasts. In contrast exogenous FGF2 18kD isoform (10-9 M) increased proliferation of both Fgf2+/+ and Fgf2-/- osteoblasts suggesting that the effect of exogenous FGF2 does not require the constitutively expressed isoforms of FGF2. To study the signal pathways, osteoblasts from Fgf2+/+ and Fgf2-/- mice were pre-treated with H89 (10-7 M), a protein kinase A inhibitor, for 1 h then treated with PGF2a or FGF2 for an additional 24h. Pre-treatment with H89 blocked the proliferative effect of FGF2 in osteoblasts of both genotype as well as the proliferative effect of PGF2a in Fgf2 +/+

osteoblasts suggesting involvement of the PKA pathway in the mitogenic response. FGF2 increased the p44/42 MAPK synthesis in Fgf2+/+ and Fgf2-/-osteoblasts, while PGF2a increased p44/42 synthesis only in Fgf2+/+ cells indicating that endogenous FGF2 was important in PGF2a activation of the MAPK pathway. Pre-treatment with PD98059 (4x10-6 M), a MEK/ERK inhibitor, blocked the mitogenic response to FGF2 in Fgf2+/+ and Fgf2-/- osteoblasts and the proliferative effect of PGF2a in Fgf2+/+ osteoblasts.This data demonstrate for the first time that the proliferative effect of PGF2a on osteoblasts is dependent on FGF2 expression. We conclude that some of the biologic responses to PGF2a in osteoblasts are dependent on FGF2 via modulation of both PKA and MAPK pathways.

69

**Organically Modified Silica Nanoparticles (ORMOSIL) as a Vector for Gene Delivery into the Brain - Mechanisms Controlling Proliferation of Neural Stem Cells**

M. K. Stachowiak,[1] D. J. Bahrali,[2] E. K. Stachowiak,[1] I. Roy,[2] I. Klejbor,[1] N. Kaur,[2] E. J. Bergey,[2] P. N. Prasad[2]; [1]Pathology and Anatomical Sciences, SUNY, Buffalo, Buffalo, NY, [2]Chemistry, SUNY, Buffalo, Buffalo, NY

The present study was undertaken to elucidate roles of FGF receptor-1 (FGFR1) in the development of neural stem/progenitor-like cells (NS/PC). Cell surface FGFR1 stimulated proliferation of cultured nondifferentiated NS/PC. In contrast, FGFR1 that translocates to the cell nucleus stimulated NS/PC differentiation and associated gene activities. To determine the role of nuclear FGFR1 in NS/PC development in the brain we used ORMOSIL nanoparticles as a novel vector for *in vivo* gene transfection. Monodispersed nanoparticles, surface functionalized with amino groups were prepared. Stereotaxic injections of nanoparticles complexed with plasmid pEGFP into mouse brain lateral ventricle allowed us to visualize the extensive transfection and expression of enhanced green fluorescent protein in NS/PC-like cells of the subventricular zone. An *in vivo* fiber-based confocal imaging showed, in live animals, the transfection and retention of viability of the cells. Transfection of a plasmid expressing the nucleus targeting form of FGFR1, but not its tyrosine kinase deleted mutant, resulted in significant inhibition of the *in vivo* incorporation of bromodeoxyuridine into DNA of the cells in subventricular zone and adjacent rostral migratory stream. Thus the nuclear FGFR1 can control the proliferation of the NS/PC in this region of the brain. The ORMOSIL nanoparticles provide an effective gene delivery platform that may be used to elucidate and control molecular gene mechanisms that regulate development of brain NS/PC. Supported by J. Oishei Foundation, NIH/NS43621, and NSF DMR0318211 and IBN9728923.

70

**The Potential Roles of Local Delivery of Insulin on Re-epithelialization during Wound Healing**

Y. Liu,[1,2] X. Zhang,[2] Z. Zhang,[2] W. Xu,[2] M. Yao,[1] M. Martins-Green[1]; [1]Cell Biology & Neuroscience, University of California Riverside, Riverside, CA, [2]Burn Department, Ruijin Hospital Shanghai Second Medical University, Shanghai, China

Topical insulin application is beneficial in the healing of ulcerations and burns. However, the underlying mechanisms of its function have not been elucidated. To investigate the effects and mechanisms of insulin on re-epithelialization during the wound healing process, deep-partial thickness scald wounds were made in rats. The time and percentage of re-epithelialization in this model were evaluated. We found that lower doses (0.1μg) of locally-delivered insulin had a significant effect on re-epithelialization while not affecting systemic glucose levels. Insulin-treated burns heal much faster (22.67±3.01d) than those that are untreated (20±1.79d); the percentage of re-epithelialized wound area is significantly increased by lower dose of insulin applied at days 9 and 13 after wounding. Furthermore, we found that many more skin appendages were found with insulin treated wounds. To further decipher the cellular and molecular mechanisms of insulin function, we analyzed the effects of insulin on keratinocyte proliferation and migration. The percentage of keratinocytes in S phase is much higher with insulin treatment than without (39.50±12.7% versus 25.63±9.37%), and the percentage of cells in G2-M phase is markedly increased with insulin treatment (0.70±0.50% versus 1.38±0.60%, $P<0.05$) as well. Insulin treatment also induces keratinocyte migration. These results suggest that local delivery of insulin stimulates DNA synthesis, mitogenesis, and migration of keratinocytes. Furthermore, our data show that insulin treatment significantly increases the epidermis growth factor (EGF) mRNA levels in the early stages of re-epithelialization. This suggests that insulin may accelerate re-epithelialization during wound healing by stimulating EGF expression. Currently, we are performing studies to determine the molecular mechanisms involved in this process.

71

**Silencing of CCR5 Blocks the *Leishmania donovani* Entry into Murine Macrophages**

S. Majumdar, S. Majumdar (Bhattacharyya), R. Dey, N. Majumder, S. Bhattacharjee; Microbiology, Bose Institute, Kolkata, India

This study explores a possible therapeutic approach against visceral leishmaniasis by the gene silencing of chemokine receptor (CCR5). C-C chemokine receptor 5 (CCR5) plays a major role for parasitic entry into the macrophages. In our present study, we attempted to inhibit *Leishmania donovani* entry by blocking the CCR5 expression in primary murine macrophages as well as macrophage like cell lines (J774) using small interfering RNA (siRNA). We directly transfected two double-stranded siRNAs, designed against mouse CCR5 gene both into control and experimental macrophage culture before *Leishmania donovani* infection. We got significant silencing of CCR5 expression at 12hrs. and 24hrs. We observed significant increases in IFN-γ and IL-12 p40 mRNA synthesis at 12 hrs and 24 hrs as evidenced by semi-quantitative PCR. These findings suggest the potential for a novel therapeutic strategy by using CCR5 siRNA transfected in macrophages to boost the Th1 response against leishmanial pathogens.

72

**Nuclear Localization of the Human Prolactin Receptor via Residue Y587 Modulates Stat5a Phosphorylation, Transactivation and Localization**

S. L. Gadd, C. V. Clevenger; Pathology, Northwestern University, Chicago, IL

Prolactin (PRL) is necessary for the proliferation and maturation of the human breast. PRL activates the human PRL receptor (hPRLr), inducing the rapid phosphorylation of hPRLr, Jak2, and Stat5a, resulting in the nuclear localization and transcriptional activation of Stat5a. We have previously shown hPRLr in the nucleus of several different cell lines using biochemical and immunohistochemical assays. We also demonstrated a PRL-inducible association between nuclear hPRLr and Stat5a. The distal tyrosine residue of the rat long PRLr is involved in Stat5a association and activation; therefore, we mutated the distal tyrosine of hPRLr to phenylalanine (Y587F-hPRLr) and assayed the effect of this mutant on Stat5a

signaling. Preliminary co-immunoprecipitation studies demonstrated decreased association between the Y587F-PRLr and Stat5a compared to wild-type hPRLr. The Y587F mutation also resulted in decreased Stat5a phosphorylation and transactivation compared to wild-type hPRLr. Interestingly, nuclear localization of both Y587F-PRLr and Stat5a was decreased in cells expressing the Y587F mutant compared to wild-type hPRLr, suggesting that hPRLr and Stat5a enter the nucleus as a complex. To determine the direct effect of nuclear hPRLr on Stat5a transactivation, we replaced the ER leader sequence with a nuclear localization signal (NLS-hPRLr) and assayed the effect of this construct on Stat5a transactivation. In cells co-transfected with NLS-hPRLr and wild-type hPRLr, Stat5a transactivation increased two-fold over cells transfected with wild-type hPRLr alone, indicating that the effect of hPRLr on Stat5a transactivation is partially due to nuclear hPRLr. These findings support a functional role for the hPRLr at both the cell surface and the nucleus.

73

**Ligand-Independent Dimerization of the Human Prolactin Receptor Isoforms: Functional Implications**

S. L. Gadd, C. V. Clevenger; Pathology, Northwestern University, Chicago, IL

Prolactin (PRL) significantly contributes to the growth and differentiation of breast cancer. PRL acts at the molecular level by activating the prolactin receptor (PRLr), a transmembrane receptor belonging to the cytokine receptor family. The accepted view has been that PRL activates the PRLr by inducing dimerization of the receptor, but recent reports show ligand-independent dimerization of other cytokine receptors. Using co-immunoprecipitation assays, we have confirmed ligand-independent dimerization of the PRLr in T47D breast cancer and HepG2 liver carcinoma cells. In addition, studies performed in mammalian cells transfected with differentially epitope-tagged isoforms of the PRLr indicated that long, intermediate and deltaS1 PRLr isoforms also formed a complex in a ligand-independent manner. To determine the domain(s) involved in PRLr ligand-independent dimerization, we generated PRLr constructs as follows: (1) the TM-ICD, which consisted of the transmembrane (TM) domain and the intracellular domain (ICD) but lacked the extracellular domain, and (2) the TM-ECD, which consisted of the TM domain and the ECD but lacked the ICD. Mammalian cells transfected with differentially epitope-tagged TM-ICD and TM-ECD mutant PRLrs demonstrated ligand-independent dimerization of these constructs, implicating a significant role for the TM domain in this process. However, these truncated PRLrs were functionally inert alone or in combination in luciferase assays. Co-transfection studies using long hPRLr and the truncated mutants showed that TM-ECD inhibited long hPRLr signaling, whereas TM-ICD potentiated long hPRLr signaling in T47D cells, suggesting that a PRLr heterodimer in which one of the receptors lacks the ECD is capable of PRL signal transduction.

74

**Use of Cell Permeant TGFα Cytoplasmic Tail Peptides to Isolate in vivo Protein Complexes Containing the PDZ Scaffolding Protein MAGI-3 in MDCK Cells**

J. L. Franklin,[1] G. Bogatcheva,[2] J. Tam,[3] A. L. Ham,[4] R. J. Coffey[5]; [1]Cell and Developmental Biology, Vanderbilt University, Nashville, TN, [2]Medicine Epithelial Biology Program, Vanderbilt University, Nashville, TN, [3]Biomedical Sciences, The Scripps Research Institute East, Jupiter, FL, [4]Department of Biochemistry and Mass Spectrometry Research Center, Vanderbilt University, Nashville, TN, [5]Cell and Developmental Biology, Medicine, Epithelial Biology Program, Cancer Center, Vanderbilt University, Nashville, TN

The multiple PDZ-domain-containing membrane associated guanylate kinase inverted (MAGI) family of scaffolding proteins, which includes three mammalian members (MAGI's 1-3), have been shown to facilitate the interaction of many protein complexes with diverse cellular functions. We have shown that MAGI-3 binds to the EGF-receptor ligand, TGFα, through MAGI-3's PDZ1 with the type I PDZ target on TGFα, increasing TGFα's efficient secretion. In addition, localization of MAGI-3 in a cellular environment depends on cell-cell interactions, where in a polarized setting MAGI-3 is found along the lateral membrane and in the cytoplasm. Cell-cell contact increases MAGI-3's ability to enhance TGFα secretion perhaps by re-localizing MAGI-3 within the cell. To isolate protein complexes that contain TGFα and MAGI-3, we have utilized a biotinylated cell permeant TGFα cytoplasmic tail peptide that contains or lacks the C-terminal PDZ target (ETVV). We show that the TGFα cell permeant peptide can enter cells and interact stably with wild-type and variant GFP-tagged MAGI-3 in MDCK cells. This interaction requires both PDZ1 of MAGI-3 and the PDZ target on TGFα. Furthermore, it can be used to precipitate MAGI-3 through the biotin moiety on the peptide and isolate MAGI-3-containing complexes from a living cell. Specific MAGI-3 associated proteins isolated by this method have been identified. Several of these proteins contain PDZ-targets and are therefore good potential MAGI-3 interacting partners in TGFα-containing complexes. We are currently validating the association of these proteins with MAGI-3 and testing their roles in TGFα trafficking secretion and processing.

75

**Growth/Differentiation Factor-15 Knockout Mice Develop with Deficits in Distinct Neuronal Populations**

J. Strelau; Neuroanatomy, University of Heidelberg, Heidelberg, Germany

Growth/differentiation factor-15 (GDF-15) is a divergent member of the TGF-beta superfamily. In the CNS the choroid plexus epithelium represents the site of strongest expression in the neonatal and adult brain. Functional studies have demonstrated neurotrophic effects of the growth factor in vitro and in vivo. To further analyse the role of GDF-15 for the development and maintenance of neurons in the CNS we generated a GDF-15 deficient mouse line, where the GDF-15 encoding DNA sequence was replaced by the lacZ gene. Using in situ hybridization and X-Gal staining we found lacZ expression in the subventricular zone of the developing spinal cord in and in the hem region of the embryonic cerebral cortex. In parallel we started to quantify the numbers of midbrain dopaminergic neurons, noradrenergic neurons in the locus coeruleus and motoneurons in different brain stem nuclei and the lumbar spinal cord. We compared 30-week-old wild-type and GDF-15 deficient mice using tyrosine hydroxylase immunohistochemistry and cresyl violet staining. We found a significant reduction of 11% in the number of TH+ neurons in the substantia nigra. Densities of striatal TH+ fibers were reduced by 11.5%. No difference was observed in the number of TH+ neurons in the locus coeruleus, indicating that reduction in TH+ fibers is due to deficits in the dopaminergic system. Notably, the number of trigeminal, facial and spinal (L1-L6) motoneurons in GDF-15 deficient mice was reduced by 20%. Oculomotor and hypoglossal motoneurons were not affected. As neonatal mice show no difference in the number of facial motoneurons we assume that the motoneuron loss occurs postnataly. Taken together we suggest that GDF-15 constitutes a novel neurotrophic factor for motoneurons and midbrain dopaminergic neurons. Supported by Deutsche Forschungsgemeinschaft (Str616/3-4 and SFB 636)

76

**The $Ca^{2+}$ Dependent Protease Calpain-1 is Responsible For the C-terminus Cleavage of Fz-7 in Endothelial Cells**

I. T. Struewing, C. D. Mao; GCNS, University of Kentucky, Lexington, KY

Frizzled receptors are a family of seven transmembrane domains G-protein-coupled receptors that are activated in particular by Wnt proteins. Once activated, they may elicit three signaling pathways: Wnt/β-catenin, planar cell polarity, or $Ca^{2+}$/Protein Kinase C. Among them, Fz-7 has been shown to be involved in cell migration during gastrulation and neuronal migration in *Xenopus* and Zebrafish development, as well as in tumor cell migration. We have found that Fz-7 is expressed in endothelial cells, and have used an Fz-7 construct dually tagged at the N and C-termini to determine its role and regulation in endothelial cells. During endothelial cell migration and adhesion Fz-7 is asymmetrically distributed at the plasma membrane as shown using immunofluorescence confocal microscopy. While full length Fz7 receptors are found in the endoplasmic reticulum(ER) and at the plasma membrane, some intracellular vesicles are only positive for the Fz7 C-terminus. Western blot analysis of Fz7 forms has revealed a novel 10 kDa C-terminus cleaved fragment that is detectable for wild-type Fz-7 and Fz-7 mutant forms trafficking to the plasma membrane, but not for Fz-7 mutant forms retained in the ER ruling out an ER-associated degradation. This Fz7 C-terminus cleavage is up-regulated by the $Ca^{2+}$ ionophore ionomycin and the PKC activator PMA, and increases with cell density. Fz-7 C-terminus cleavage is decreased by the calpain inhibitors calpeptin and Z-LL-CHO. Co-transfection with a dominant negative form of Calpain-1 greatly reduces both basal and PMA-stimulated levels of Fz-7 C-terminus cleavage. Altogether these results reveal that the C-terminus cleavage of the Fz-7 receptor is regulated by $Ca^{2+}$-dependent events, such as cell adhesion, and is dependent on Calpain-1 activity. This specific C-terminus cleavage may represent a down-regulation mechanism of Fz-7 receptors at the plasma membrane after activation.

77

**OGF-OGFr Axis and Its Inhibitory Actions on Cell Cycle Progression of Head and Neck Cancer**

F. Cheng,[1] I. S. Zagon,[1] M. F. Verderame,[2] P. J. McLaughlin[1]; [1]Department of Neural and Behavioral Sciences, The Pennsylvania State University College of Medicine, Hershey, PA, [2]Department of Medicine, The Pennsylvania State University College of Medicine, Hershey, PA

Opioid growth factor (OGF) is an endogenous opioid peptide ([$Met^5$]-enkephalin) that interacts with the OGF receptor (OGFr), and serves as a tonically active negative growth factor in neoplasia. Previous studies showed that OGF inhibits the growth of human squamous cell carcinoma of the head and neck (SCCHN) *in vitro* and *in vivo*, and is targeted to cell proliferation. To clarify the mechanism by which OGF inhibits cell replication, we investigated the effect of OGF on cell cycle activity in the SCCHN cell line SCC1. In cultures synchronized with nocodazole, flow cytometry revealed that OGF treatment resulted in fewer cells exiting the G1 phase of the cell cycle in comparison to controls. Focusing on the role of OGF in the G1 to S phase transition, OGF decreased the phosphorylation of retinoblastoma protein (Rb) without changing the total Rb expression. This change was correlated with reduced cdk4 kinase activity while the total cdk4 expression did not change. OGF treatment induced cyclin-dependent kinase inhibitor (CKI) p16 protein expression but had no effect on p21 or p27 protein expression. Short-term blockade of OGF-OGFr interactions with the short-acting opioid antagonist, naloxone (NAL), revealed that increased expression of p16 protein by OGF was completely abolished by NAL. Inhibition of p16(INK4a) activation by p16 specific siRNA blocked OGF inhibiting action on SCC1 cells. Collectively, these results indicate that the receptor-mediated, growth inhibitory effects of the OGF-OGFr axis in SCC1 cells are associated with induction of p16 expression, that in turn, repress the activity of cyclin D1/cdk4 and Rb phosphorylation.

78

**The OGF-OGFr Axis is a Determinant of Corneal Wound Healing: Molecular Perturbations and Repercussions**

I. S. Zagon,[1] J. W. Sassani,[2] M. F. Verderame,[3] P. J. McLaughlin[1]; [1]Neural and Behavioral Sciences, H109, Penn State University, Hershey, PA, [2]Ophthalmology, Penn State University, Hershey, PA, [3]Medicine, Penn State University, Hershey, PA

Blockade of OGF from the OGF receptor by the opioid antagonist naltrexone (NTX) results in acceleration of corneal re-epithelialization in rat, rabbit, and human. To test whether this pharmacological effect of NTX is related directly to the OGF-OGFr axis, biolistic DNA delivery by particle-mediated gene transfer using a gene gun was utilized to deliver antisense OGFr gene to the corneal epithelium of adult rats. Disruption of the OGF-OGFr axis would be predicted to accelerate corneal wound healing if this peptide and receptor complex played a direct role in re-epithelialization. The plasmid pcDNA3.1-OGFr, carrying the rat OGFr cDNA under the control of the CMV immediate early promoter, was delivered twice at 300 psi into one eye of each rat (AS rats); empty vectors were transfected into one eye of another group of animals (EV rats). This regimen resulted in >90% transfection of the central and peripheral corneal epithelium without penetration into the stroma. After a 24-hr latency, a corneal abrasion was created using a 3 mm trephine. Corneal wounds were tracked photographically with fluorescein dye, and images quantitated using Optimas software. At 16 hr after wounding, the residual defect was 40% smaller for AS rats than for EV rats ($p<0.05$). After 24 hr, the defect in rats transfected with antisense was 98% smaller than for EV animals ($p=0.03$). Rats injected with BrdU to determine DNA synthesis rates showed significantly a greater number of BrdU labeled cells in AS rats relative to EV animals. These data reveal that the OGF-OGFr axis is crucial to determining the outcome of corneal wound healing. Moreover, gene gun technology supports the use of gene therapy for ocular diseases.

79

**Salvianolic Acid B and Magnolol Inhibit Vascular Smooth Muscle Cell Proliferation and Migration by Stromal Cell-Derived Factor-1 Alpha**

C. J. Wu, C. Chen; Biological Science and Technology, China Medical University, Taichung, Taiwan Republic of China

Atherosclerosis is a disease with complex processes in which monocytes and lymphocytes are recruited form the blood into the arterial intima. Stromal cell-derived factor-1alpha (SDF-1alpha) and its unique receptor, CXCR4, have been recently implicated in the development of neoitimal formation after vascular injury in apolipoprotein E-deficient mice. In the present study we investigated two Chinese herbal extracts, salvianolic acid B and magnolol, about their inhibitory effects on rat vascular smooth muscle cells (VSMCs) for cells proliferation, CXCR4 expression, cellular migration and its related signal pathway. Our results demonstrated that SDF-1alpha-induced cell proliferation can be significantly inhibited by salvianolic acid B and magnolol. Cell migration potentiated by SDF-1alpha was also attenuated by salvianolic acid B and magnolol. In the aspect of apoptosis, salvianolic acid B and magnolol can induce VSMCs apoptosis via caspase 3 up-regulation or Bcl-2 down-regulation. In summary,

considering their effects on cell proliferation and migration, salvianolic acid B and magnolol may play an important role in preventing atherosclerosis and balloon injury-induced neoitimal formation.

80

### RAGE Engagement in Myoblasts Modulates Proliferation, Apoptosis, Adhesiveness, Migration and Invasiveness

G. Sorci, F. Riuzzi, R. Donato; Dept Exp Med & Biochem Sci, University of Perugia, Perugia, Italy

We reported that RAGE (receptor for advanced glycation end products), a multiligand receptor of the immunoglobulin superfamily expressed in myoblasts, when activated by its ligand amphoterin (HMGB1), stimulates rat L6 myoblast differentiation via a Cdc42-Rac-MKK6-p38 MAPK pathway, and that RAGE expression in rat skeletal muscle tissue is developmentally regulated (1). We show here that inhibition of RAGE function via overexpression of a signaling-deficient RAGE mutant (RAGEΔcyto) results in increased L6 myoblast proliferation, migration and invasiveness, and decreased apoptosis and adhesiveness, while myoblasts overexpressing RAGE behave the opposite, compared with mock-transfected myoblasts. These effects are accompanied by a decreased induction of the proliferation inhibitor $p21^{WAF1}$ and expression of adhesion molecules known to be important for myogenesis, increased induction of cyclin D1, extent of Rb, ERK1/2 and JNK phosphorylation and activity of matrix metalloproteinases 1 and 2 in L6/RAGEΔcyto myoblasts, while the opposite occurs in L6/RAGE myoblasts, compared with mock-transfected myoblasts. Neutralization of culture medium amphoterin negates all effects of RAGE activation, suggesting that amphoterin is the RAGE ligand involved in the RAGE-dependent effects in myoblasts. Effects of RAGE signaling in myoblasts appear to depend on p38 MAPK activation only, because the p38 MAPK inhibitor, SB203580, reduces them in both L6/RAGE and L6/mock myoblasts, and a similar reduction is observed in L6/RAGE and L6/mock myoblasts transiently transfected with MKK6AA, an inactive mutant of the upstream p38 MAPK, MKK6. Thus, the amphoterin/RAGE pair stimulates myoblasts differentiation by the combined effect of inhibition of proliferation and stimulation of differentiation. Also, based on the findings reported here, we speculate that deregulation of RAGE expression and/or function might contribute to the increased motility and invasiveness of myoblast neoplastic counterparts.1. Sorci G. et al. (2004) Mol. Cell. Biol. 24:4880-4894.

81

### Complementary Roles of IL-8 and VEGF in Inflammation, Angiogenesis and Healing

M. Yao, M. Petreaca, M. Martins-Green; Cell Biology & Neuroscience, UCR, Riverside, CA

Inflammation is invariably accompanied by angiogenesis but it is not known how angiogenic factors present during the inflammatory phase of wound healing, are involved in inflammation-induced angiogenesis. Here, we address the contribution of two such factors, interleukin-8 (IL-8/CXCL8) and VEGF, in this process. IL-8 levels increase rapidly after wounding in rabbits, reaching maximal levels at 24hrs, whereas VEGF peaks at 3 days. The time course of the infiltration of inflammatory cells and production of new blood vessels after wounding was also determined. Neutrophils were markedly increased 4hr after wounding, and their level peaked at 24hr. Monocyte/macrophage infiltration peaked at 48hr and decreased by day 4. The number of blood vessels was significantly increased by day 3, continued to increase through day 5 and peaked between days 7 and 14. In order to determine the contributions of IL-8 and VEGF to inflammation and angiogenesis, we performed the wounding experiments in the presence and absence of their antibodies or antibodies/inhibitors to their receptors. Inhibition of IL-8 or its receptors blocked development of early stage microvessels but did not inhibit late-stage vessels; in contrast, VEGF/VEGFR antibodies blocked the late stage without altering the early stage. Furthermore, whereas inhibition of IL-8 resulted in decrease of infiltration of inflammatory cells, inhibition of VEGF resulted in significant increase in macrophages in the wound tissue. We found that VEGF induces apoptosis of macrophages in human cultures. Using FACS analysis and Annexin-V antibodies, we show that the number of dead cells in VEGF-treated macrophages was significantly higher than control and that the maximum effect occurred at 24hrs after treatment. We are currently investigating the signaling pathways involved. Our results suggest that IL-8 and VEGF play complementary roles in inflammation and angiogenesis during wound healing.

82

### A New Synthetic Tripeptide that Promotes Cell Activation in Epidermal Cells

N. Domloge, K. Cucumel, J. Botto, C. Dal Farra; Vincience, Sophia antipolis, France

When cell membrane-related receptors are activated, they trigger a second messenger responsible for a subsequent cascade of cellular responses. This general process is one of the most important mechanisms of cell activation and signal transduction. To understand further the relationship between cell activation and cell aging, we developed a synthetic tripeptide, and tested its effects on cell activation. Cellular ATP assessment of human fibroblasts treated with 1% of the peptide, revealed an increase in cellular ATP level. This increase started within 5 minutes, and reached its maximum 15 minutes after the peptide was administered. Together with the peptide structure, this activation suggests that the peptide directly stimulates APnA (ATP reservoir molecules) receptors in the cells. Moreover, as Ca2+ plays a central role in cell signalling, we also evaluated the effect of the peptide on cellular Ca2+ level. Using a fluorescent probe and confocal microscopy, we monitored Ca2+ expression in selected single HaCaT cells in response to peptide administration. The selected cells elicited maximum fluorescence intensity 90 s after the peptide was administered, and dropped down to basal level thereafter. Further studies on keratin synthesis confirmed these results and additional tests on *in vitro* wound repair and protein synthesis confirmed general cell activation by the new peptide. In addition to the tripeptide's great similarity to the described APnA receptor, these results strongly suggest that the structure of the tripeptide is responsible for ATP induction. The peptide also shows evidence of cell activation via other G-protein-coupled receptors in epidermal cells. Within the context of topical skin regenerating and energizing products, these set of studies demonstrate that the new tripeptide possesses an effect on cell activation that can be of great use.

## Signal Transduction I (83-104)

83

### Ric-8B, a Potential Regulator for Galpha-olf

L. von Dannecker, A. F. Mercadante, B. Malnic; Departamento de Bioquímica, Instituto de Química - USP, São Paulo, Brazil

The olfactory system is able to recognize and discriminate a vast array of chemical structures. Odorants activate specific odorant receptors (ORs) expressed in the cilia of olfactory neurons. As a consequence Galpha-olf is activated and the olfactory neurons are depolarized via cyclic-AMP

increase. In order to search for potential regulators for Galpha-olf we used yeast two-hybrid to screen an olfactory epithelium cDNA library with Galpha-olf as bait. We found that Ric-8B, a putative GTP exchange factor, is able to interact with Galpha-olf. We performed Northern blot and RT-PCR experiments to examine the tissue distribution of Ric-8B expression. Our data show that Ric-8B gene is preferentially transcribed in the olfactory epithelium and, to a lower extent, in the brain, eye, and muscle. We performed *in situ* hybridization experiments to determine the localization of Ric-8B and Galpha-olf in the olfactory epithelium and found that they are coexpressed in mature olfactory neurons. Recent experiments showed that expression of Ric-8B in the olfactory epithelium follows Galpha-olf time course expression profile with the initial detection for both genes in embryonic stages at prenatal day 16 (E.16). We also performed *in situ* hybridization to determine the distribution of Ric-8B mRNA in the brain. Ric-8B mRNA was colocalized with that of Galpha-olf, except for the Purkinje cells in the cerebellum. To verify whether Ric-8B is able to regulate the activity of Galpha-olf, we transfected HEK293 cells with the expression vectors for Galpha-olf, Ric-8B and the receptors Beta-2-AR or D1R. Our results show that Ric-8B is able to potentiate the ability of Galpha-olf to activate cAMP production if receptors were previously activated. The potentiation of Galpha-olf by Ric-8B and their highly restricted and colocalized expression patterns strongly indicate that Ric-8B is a potential regulator for Galpha-olf.

84

**Actin Cytoskeleton-dependent Control of Rho Activation: Role of p190 RhoGAP and Filamin**

A. Mammoto, S. Huang, D. E. Ingber; Vascular Biology Program, Children's Hospital/Harvard Medical School, Boston, MA

Cell shape-dependent control of cell cycle progression plays an important role in the spatial heterogeneity of proliferation that drives tissue morphogenesis. We previously showed that the small G protein Rho mediates cell shape-dependent control of cell cycle progression in human capillary endothelial (HCE) cells (Mammoto et al. J.Biol.Chem. 279:26323, 2004). Rho activation by growth factors has been intensively studied, however, little is known about how cytoskeletal distortion regulates Rho activity. Rho activity is increased in adherent HCE cells that were induced to round up by treatment with the actin cytoskeletal disruptor, cytochalasin D (cytoD) or by placing the cells in suspension, whereas it remained low in spread adherent cells. To elucidate the mechanism by which Rho activity senses cell shape we now show that the Rho GTPase-activating protein, p190RhoGAP, which inactivates Rho, conveys the effects of cytoskeletal configuration on cell shape on Rho activity. p190RhoGAP was translocated to the lipid raft fraction when cells were spread, whereas this translocation was inhibited in suspended cells and cytoD-treated cells. p190RhoGAP tyrosine phosphorylation appeared within the raft fraction in spread cells. Interestingly, cell rounding fails to exclude p190RhoGAP from the rafts and to increase Rho activity in cells that lack filamin. These data indicate that changes in the structural configuration of the actin cytoskeleton impact Rho activity by controlling p190RhoGAP translocation to lipid rafts and that filamin represents a central control point in this pathway. This finding provides a handle to study how a macroscopic parameter, cell shape, is translated into intracellular biochemical signals that govern how cells make cell fate decisions in the physical context of the tissue.

85

**GTP-like Conformation of GDP-bound Arl10b GTPase**

A. Atanassova,[1] J. Choe,[1] S. Dimov,[1] Y. Shen,[1] A. Botchkarev,[1] J. Lew,[1] I. Kozieradzki,[1] H. Park[2]; [1]Structural Genomics Consortium, University of Toronto, Toronto, ON, Canada, [2]Structural Genomics Consortium, Department of Pharmacology, University of Toronto, Toronto, ON, Canada

ADP-ribosylation factor (ARF) proteins are Ras-like small G proteins with the molecular weight of 20 kDa. They include ARF, Arf-like (ARL) and Secretion-associated and Ras-related (SAR) proteins, all of which are collectively called the ARF family proteins. The GDP/GTP (inactivation/activation) cycle of the ARF family proteins has been known to cause conformational changes at the N-terminal helix as well as the nucleotide-interacting switch 1 and 2 regions. Here, we solved the structure of ARL10B protein, which contains a truncation of the N-terminal helix, by the x-ray crystallographic technique. The truncation of the N-terminal helix mimics its interactions with lipid membrane. The structure shows an active conformation similar to the GTP-bound state of other ARF proteins although ARL10B is still bound to GDP. This finding suggests that the N-terminal helix interactions with lipid membrane, not the GDP-GTP exchange, trigger the conformational switch of ARL10B from the inactive to the active state. Important implication of this activation mechanism is that the activation of ARL10B proceeds with a conformational switch to the GTP-like state via the N-terminal helix recruitment to membranes, leading to the GDP-GTP exchange by a classical guanine nucleotide exchange factor.

86

**A Ciliary Sensation: Mapping Components of the GTP Signaling Pathway in *Tetrahymena thermophila***

H. G. Kuruvilla; Science and Mathematics, Cedarville University, Cedarville, OH

*Tetrahymena thermophila* are an excellent model system in which to study chemokinesis because of their ease of growth in culture as well as their adaptability to behavioral and biochemical assays. Recent work by Christensen *et al.* (2003), as well as Csaba *et al.* (2004) suggests that the cilia of this organism may actually serve as signaling apparatus, allowing for chemokinesis to take place. Both of these prior studies were done with chemoattractants. In our current study, we have used immunofluorescence labeling to map components of the GTP chemorepellent pathway in *Tetrahymena*. Our data show that many enzymes involved in the chemorepellent pathway, including tyrosine kinase and PP2A, immunolocalize to the cilia. These data further confirm the hypothesis that cilia may serve as signaling organelles in *Tetrahymena*, in addition to their roles in motility and chemokinesis.

87

**Snapin, a SNARE-Associated Protein, Interacts with the Type VI Adenylyl Cyclase at Postsynaptic Sites**

C. J. Wu, J. Chou, A. C. Hung, H. Chang, C. Huang, H. Lai, Y. Chern; Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan Republic of China

Snapin is a binding protein of SNAP25 (synaptosome-associated protein -25), a component of the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) complex. It has been suggested to be important in mediating neurotransmitter release process through its modulation of the interactions between the SNARE complex and synaptotagmin. In the present study, we identified Snapin as a novel interacting protein of type VI adenylyl cyclase (ACVI) by yeast-2-hybrid screening. Mutational analysis showed that the interaction domains of

ACVI and Snapin were located within amino acids 1-86 of ACVI and 33-51 of Snapin, respectively. Expression of Snapin specifically eliminated protein kinase C (PKC)-mediated suppression of ACVI, while it had no effect on cAMP-dependent protein kinase (PKA) or calcium mediated suppression of ACVI. Since the cAMP-dependent pathway has been implicated in neurotransmitter release, and phosphorylation of Snapin by PKA enhances its interaction with the SNARE complex, direct interactions between Snapin and ACVI may facilitate the release process induced by cAMP. Immunofluorescence staining showed co-localization of Snapin and ACVI in primary hippocampal neurons. Using MAP2 as a somatodendritic marker, the ACVI/Snapin complexes were located in somas and proximal dendrites. In addition, we showed that ACVI/Snapin complexes also co-localized with 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors, and postsynaptic density-95 protein (PSD-95). This observation suggests that, apart from presynaptic regulation of neurotransmitter release, the ACVI/Snapin interaction may be functionally important at postsynaptic sites. Together, these data suggest that the ACVI/Snapin interaction might play a role in cAMP-mediated neuronal plasticity.

88

**Understanding the Conformational Change in a Protein Kinase C Probe**

C. Jost, J. Brumbaugh, A. Schleifenbaum, A. Guinan, T. Skrahina, C. Schultz; Gene Expression, EMBL Heidelberg, Heidelberg, Germany

We recently developed a FRET based probe (KCP-1) which monitors PKC activity in living cells. (Schleifenbaum *et al., J. Am. Chem. Soc.*, 2004) The probe is based on pleckstrin, the major PKC substrate in platelets, and changes its conformation upon phosphorylation. The conformational change in the construct EYFP-PH1-substrate-DEP-GFP$^2$ is indicated by a change in emission ratio between the two fluorophores. To understand the nature of the conformational change, we studied two regions of KCP-1 more intensively: the N-terminal linker region between pleckstrin and YFP and the C-terminal linker region between pleckstrin and GFP$^2$. The C-terminal linker region of KCP-1 contains a stretch of acidic amino acids (acidic loop). By deleting this acidic loop we obtained a new probe (KCP-2) which showed the reverse effect to KCP-1: the FRET ratio decreased by 20-40% instead of an increase. Subsequent systematic studies of the linker region showed that most charged residues were essential for the performance of KCP-1. In addition, other crucial residues were identified, e.g. V235. Elongation of the linker region had little effect on KCP-1. Similar elongations or shortening of the N-terminal linker region of the PH domain showed that KCP-1 was very sensitive to any kind of alteration, while KCP-2 was unaffected. For example, the KCP-1 signal was reduced to 20% of the original with 6 additional amino acids, while KCP-2 still showed up to 80% activity. We conclude that in KCP-1, both the acidic loop and the N-terminal linker region are involved in producing the complex conformational change induced by phosphorylation. On the other hand, the shorter probe, KCP-2, seems to rely entirely on the interaction between the PH1 and DEP domains of pleckstrin.

89

**Interaction between PKC and HSP 70 in Fibroblasts Overexpressing Cellular Ras**

C. O'Brien, S. K. Murphy; Biological Sciences, University of the Sciences in Philadelphia, Philadelphia, PA

Heat shock proteins are a conserved group of proteins involved in cell regulation. Constitutively produced HSP 70 acts as a molecular chaperone. HSP 70 can also be induced by environmental stressors. Overexpression of HSP 70 has been shown to increase resistance to apoptosis through interaction with signaling pathways, possibly implicating HSPs in carcinogenesis. Previously we showed that fibroblasts overexpressing cellular Ras had elevated HSP 70 relative to controls. Ras expressing cells exhibited a transformed phenotype that was reversed by inhibition of HSP 70. Further, Ras expressing cells show increased activity of $PIP_2$-PLC. HSP 70 inhibition did not alter this, suggesting HSP 70 acted downstream of $PIP_2$. Protein kinase C (PKC) plays a role in growth regulation and cytoskeletal organization. Additionally, PKC phosphorylates HSF-1 which is essential for HSP 70 transcription. We propose that PKC and HSP 70 are involved in a positive feedback loop in which PKC phosphorylates HSF-I inducing HSP 70. In turn, HSP 70 prevents down regulation of PKC. Further, that this feedback works to regulate Ras function. To that end, we have shown that activation of PKC resulted in a 100% increase in HSP 70. Conversely, inhibition of PKC led to a 40% decrease in HSP 70 expression. To further support our hypothesis we have shown a correlation between elevated PKC activity, increased expression of HSP 70 and the transforming capabilities of Ras. Concomitant with an increase in PKC activity and subsequent increase in HSP 70 expression we have observed an increase in $^3$H-thymidine incorporation and disruption of the cytoskeleton. These conditions were reversed in the presence of both PKC and HSP 70 inhibitors. These data suggest that PKC and HSP 70 interact via positive feedback and contribute to the ability of Ras to transform cells.

90

**Studies on the Cell Activation and Signaling Properties of Diacylglycerol-Rich Extract**

C. Dal Farra, E. Bauza, C. Coquet, N. Domloge; Vincience, Sophia antipolis, France

PKC phosphorylation and activation by diacylglycerol (DAG) induces a variety of essential cellular responses. This mechanism results from an important cellular signaling pathway involving tyrosine kinase receptor activation. In this area, we developed a new DAG-rich extract in an effort to induce direct and rapid cell activation. In these studies, we tested the effect of DAG-rich extract on cultured human epidermal cells and fibroblasts, and confirmed the consequential activation of terminal differentiation following cell activation by the extract. First, immunofluorescence studies on protein expression were performed. These studies revealed that application of the DAG-rich extract to fibroblasts enhanced fibronectin, collagen I, and collagen III synthesis. Second, studies on HaCaT cells showed that DAG-rich extract-treated cells exhibited enhanced beta 1 integrin expression. HaCaT studies also revealed an increase in filaggrin expression, a marker of terminal differentiation. This increase in filaggrin was found in a dose-dependent manner. Immunoblotting studies demonstrated that PKC activation by DAG-rich extract is accompanied by an increase in the expression of differentiation markers and by an increase in protein synthesis. Studies on ex vivo skin confirmed these findings. Interestingly, ATP evaluation studies revealed that administration of the extract resulted in an increase in cellular ATP level. Moreover, studies on Ca2+ expression in selected single cells showed an increase in Ca2+ in response to extract administration. These results studies lend strong support to the hypothesis that in addition to PKC, DAG-rich extract activates other cell targets as well.Taken together, these studies demonstrate important properties possessed by DAG-rich extract through its combination of different essential cell activation and signaling mechanisms. In the context of energizing and anti-aging skin care products, these results reveal that DAG-rich extract is an active ingredient that can be of great use.

91

**A Comparison of a Novel Serum and Glucocorticoid Inducible Kinase 1 (SGK 1) Isoform within Human Keratinocytes**

B. A. Hall, S. Blakeley, T. R. Jackson; Dermatological Sciences, University of Newcastle Upon Tyne, Newcastle Upon Tyne, United Kingdom

Serum and Glucocorticoid inducible kinases (SGKs) form a group of serine/threonine kinases implicated in the transduction of cell survival and proliferation signals to the nucleus. Although mechanisms of regulation of SGK activation and expression in response to extracellular stimuli are not well understood [1]. SGKs encode a kinase domain 54% similar to the well studied kinase, Akt. As with the closely related homologue Akt, SGKs are thought to depend on phosphoinositide-3 kinase (PI-3K) for enzymatic activation. Both kinases require phosphorylation of two regulatory sites at specific Thr and Ser residues by phosphoinositide-dependent protein Kinases 1 and 2 (PDK1 and PDK2) [2]. Initial aims were to charactise the expression of SGKs within primary human keratinocytes and assess their subcellular localisation. Initial data has identified specific subcellular structures in which SGK1 localises. By use of Fluorescence Recovery After Photobleaching (FRAP) we have investigated the dynamics of eGFP-SGK1 associated with these structures in living cells. Furthermore, herein we report the discovery of a novel SGK1 variant. The use of western blotting and molecular cloning techniques confirms the serum and glucocorticoid inducibility of this variant. (1) Park et al., 1999. *The EMBO J.* 18, 3024-3033. (2) Kobayashi and Cohen, 1999. Biochem. J. 339 (319-328)

92

**Cell-Based Assays for the Simultaneous Monitoring of the Akt Pathway by Multiplex Bead Immunoassay**

T. Hager, W. Zheng, J. DeSimone, C. Fountain, J. Wang, K. Reagan; BioSource International, Camarillo, CA

The insulin receptor (IR)/IGF-1R - Akt signaling pathway is crucial for regulating cell survival, proliferation and a number of metabolic effects of insulin. Perturbations in this pathway are suspected of being key indicators and perhaps the root cause of diseases such as cancer and diabetes. Several important proteins along the pathway have been identified and their activation state monitored by detecting phosphorylation status of specific sites. A method to measure the activity of these proteins simultaneously will significantly expedite the drug discovery process targeting this pathway. In this study, MCF7 and 3T3L1 cells were treated with IGF-1 or insulin at different time intervals. The phosphorylation states of seven proteins (IR [pYpY1162/1163], IGF-1R [pYpY1135/1136], IRS-1 [pS312], Akt [pS473], GSK-3β [pS9], PRAS40 [pT246], p70S6K [pTpS421/424]) were monitored concurrently using a multiplex bead immunoassay. The results illustrate that insulin and IGF-1 activate different components along the Akt pathway in a time-dependent manner. Prolonged treatment of cells with insulin and IGF-1 induces a negative feedback regulation through phosphorylation of IRS-1 at Serine 312. The inhibitory effects of the PI3 kinase inhibitor, wortmannin, and Akt inhibitors, SH-5 and SH-6, on insulin and IGF-1 signaling were observed, showing the down-regulation of Akt [pS473], GSK-3β [pS9], PRAS40 [pT246] and p70S6K [pTpS421/424]. Our results demonstrate that simultaneous monitoring of insulin/IGF-1 signaling through downstream members of the Akt pathway allows a more complete cell-based analysis of relevant, disease-related signal transduction mechanisms and provides a valuable tool for research and drug discovery.

93

**Direct Regulation of Cell Cycle Proteins by Transforming Growth Factor-β**

D. V. Kielley, J. J. E. Doré; Basic Medical Science, Memorial University of Newfoundland, St. John's, NF, Canada

**Introduction:** Transforming growth factor β (TGF-β) causes growth stimulation in fibroblasts, and growth inhibition in epithelial cells. To explain how TGF-β exerts different growth effects, we investigated the phosphatidylinositol 3-kinase (PI3K)/ Akt pathway. We propose that PI3K signalling is partially responsible for different phenotypic effects of TGF-β on mesenchymal and epithelial cells, in conjunction with the previously described Smad pathway. **Methods**: Western blotting was used to describe temporal changes (0-3 hours) in endogenous phosphoinositide-dependent kinase (PDK-1), Akt, Glycogen Synthase Kinase-3β (GSK-3β) and Cyclin D1 phosphorylation following TGF-β2 stimulation of normal epithelial and fibroblast cell lines. **Results:** TGF-β stimulation increased PDK-1, Akt and GSK-3β phosphorylation in fibroblasts over time, while it decreased PDK-1, Akt and GSK-3β phosphorylation in epithelial cells over the same time. The GSK-3β substrate, Cyclin D1, exhibited decreasing phosphorylation in fibroblast cells and decreased protein levels of Cyclin D1 in epithelial cells. In fibroblasts, TGF-β increased Cyclin D1 complex formation with CDK-4 and nuclear localization. Conversely, TGF-β decreased complex formation in epithelial cells. TGF-β stimulated Cyclin D1/CDK complex formation was blocked by a PI3K inhibitor. **Conclusions:** In total, these results define a novel pathway for TGF-β regulation of cell cycle through PI3K/Akt in normal cells. This signalling pathway also differentiates epithelial and fibroblast cell responses to TGF-β stimulation. In conjuction with Smads, this pathway could account for the cell type specific growth responses to TGF-β. Potential alterations of this pathway may be a mechanism by which some cancers elude the normal growth regulatory system imposed by TGF-β.

94

**Insulin-stimulated Interaction between Insulin Receptor Substrate 1 and p85α and Activation of Protein Kinase B/Akt Require Rab5**

X. Su, P. D. Stahl; Department of Cell Biology and Physiology, Washington University, St. Louis, MO

Insulin resistance represents the leading factor for the development of type 2 diabetes. Binding of insulin to the insulin receptor (IR) initiates a cascade of protein phosphorylation events that lead to the activation of multiple distinct signaling pathways. Previous studies suggested that receptor endocytosis and trafficking affected different receptor signaling pathways. The small GTPase Rab5 is required for receptor-mediated endocytosis at multiple sites including the fusion of newly formed vesicles with early endosomes. Three Rab5 isoforms have been identified. Herein, we exploited the RNA interference (RNAi) technique to specifically knock-down Rab5 isoforms to determine the function of Rab5 in distinct insulin signaling pathways. siRNA against individual Rab5 isoforms had no effect on Akt and MAPK activation by insulin in NIH 3T3 cells over-expressing human IR (NIH 3T3/hIR). However, knockdown of all three Rab5 isoforms dramatically inhibited Akt activation by insulin without affecting MAPK activation. This inhibition of Akt activation was due to the impaired interaction between insulin receptor substrate1 (IRS1) and the p85α subunit of PI3K, which is known to be activated by insulin. These results indicate that Rab5 is required in the recruitment of p85α by IRS1. Interestingly, expression levels of all three Rab5 isoforms are dramatically down regulated in white adipocyte tissues (WAT) of fatty Zucker rats. Collectively, the present study suggests the involvement of Rab5 and endocytosis in obesity-induced insulin resistance and diabetes.

95

**PRC17, a Rab5 GTPase Activating Protein, Alters the Normal Trafficking and Signaling of EGFR**

M. Wainszelbaum, D. Hodzic, C. Kong, A. Charron, P. D. Stahl; Cell Biology & Physiology, Washington University Medical School, St. Louis, MO

PRC17 (Prostate cancer Chromosome 17) is a "primate specific" oncogene found in chromosome 17 that is over-expressed in selected metastatic prostate, breast tumors and tumor cell lines. It interacts with Rab5 and has Rab5 GTPase activating protein (GAP) activity. Rab5 plays a key role in vesicular trafficking from the plasma membrane to the sorting endosomal compartment. Moreover, Rab5 is involved in both Epidermal Growth Factor Receptor (EGFR) signaling and trafficking. Here we studied the molecular biology of PRC17 and the effects of the protein on EGFR mediated endocytosis and signaling. Examination of the human genome database indicates that PRC17 gene exists as 8 copies with high homology encoding isoforms that vary in a small number of amino acids nested in the GAP domain. The relative tissue abundance of PRC17 was estimated by Semi-quantitative PCR from a set of human cDNAs. Expression levels of PRC17 were comparable among the tissues tested with the exception of skeletal muscle that exhibited a low abundance. By RFLP we identified the expression of at least three isoforms and two spliced variants in those tissues. Over-expression of two different isoforms of PRC17 in cultured cells, using a retroviral system, resulted in accelerated cellular growth and in enhanced proliferation in response to EGF stimulation. MAP kinase and PKB/Akt activation responses to EGF in PRC17 transfected cells was substantially greater both in magnitude and in duration. Furthermore, EGF receptor binding and internalization was substantially increased in PRC17 transfected cells. Lastly, we found a substantial delay in EGF receptor degradation following receptor internalization in cells expressing PRC17. Our results suggest that PRC17 enhances growth factor receptor signal transduction by altering the normal trafficking of internalized receptors.

96

**Role of the Gab1 / PI3K Complex in Secretion of Glycoprotein**

B. Shi, S. Ihara, Y. Fukui; Departmemt of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan

Signet ring cell carcinoma is one of poorly differentiated adenocarcinomas and characterized by loss of cell-cell interaction and enhanced secretion of glycoproteins, such as MUC1 and MUC4. In many of these cell lines, the ErbB2/ErbB3 complex is constitutively activated. Gab1 has been shown to be phosphorylated on tyrosine by the ErbB2/ErbB3 complex. We found that Gab1 is activated and bound to phosphoinositide 3-kinase (PI3K) in such a cell line, NUGC4. Treatment with an inhibitor for PI3K, LY294002, significantly suppressed the expression of glycoproteins on the cell surface in these cells. These results were also true in other signet ring cell carcinoma lines. Knockdown of Gab1 by RNAi suppressed the expression of glycoproteins on the cell surface. The glycoprotein was found inside the cells, suggesting that it is not production but is secretion, which is inhibited by knockdown of Gab1. Expression of a RNAi-resistant Gab1 restored the suppressed secretion of glycoproteins, confirming that the effect was indeed due to knockdown of Gab1. A mutant Gab1 incapable of binding to PI3K failed to release the suppression, suggesting that binding of Gab1 to PI3K is important for secretion of glycoproteins. These results suggest that Gab1 and PI3K may play crucial roles in regulating glycoprotein secretion.

97

**Effects Of $IP_3$ Receptor Kinetics and Intramembrane Diffusive Transport on Signal Localization and Amplification in Cerebellar Purkinje Cell Spines**

N. Hernjak, I. I. Moraru, J. C. Schaff, I. Novak, B. M. Slepchenko, F. Gao, L. M. Loew; Cell Biology, University Connecticut School of Medicine, Farmington, CT

Modeling and simulation of the calcium signaling events that precede long-term depression of synaptic activity in cerebellar Purkinje cells are performed using the Virtual Cell biological modeling framework (http://vcell.org/). It is found that the unusually high density and low sensitivity of inositol-1,4,5-trisphosphate ($IP_3$) receptors are critical to the ability of the cell to generate and localize a calcium spike in a single dendritic spine. The results also demonstrate the model's capability to simulate the supralinear calcium spike observed experimentally during coincident activation of the parallel and climbing fibers. The sensitivity of the calcium spikes to certain biological and geometrical effects is investigated as well as the mechanisms that underlie the cell's ability to generate the supralinear spike. The sensitivity of calcium release rates from the $IP_3$ receptor to calcium concentrations, as well as $IP_3$ concentrations, allows for the calcium spike to form. The diffusion barrier caused by the small radius of the spine neck is shown to be a functional switch, as a threshold radius is observed above which a spike cannot be formed. The calcium buffer capacity and diffusion rates from the spine also are critical in shaping the calcium spike. Additionally, detailed spatial simulations using the new lateral membrane diffusion capabilities in the Virtual Cell addressed the issue of phosphatidylinositol 4,5-bisphosphate resuply by signal amplification of lateral membrane diffusion versus activated local de novo synthesis. Supported by NIH P41-RR013186.

98

**A Modulatory Role for Phosphoinositide 3-Kinase in Gastric Parietal Cell Stimulation**

S. E. Mettler, S. Ghayouri, G. Patangay, J. G. Forte; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

The role of phosphoinositide 3-kinase (PI3K) in the stimulation of acid secretion by gastric parietal cells has been examined. Previous work has shown that histamine (H) stimulates glands ~3.5 fold over resting levels and histamine plus IBMX (H+I) stimulates glands ~8 fold over resting using the standard aminopyrine (AP) assay. Increased stimulation by IBMX has been attributed to inhibition of phosphodiesterase resulting in increased [cAMP]. Here, gastric glands were treated with H plus a common PI3K inhibitor, LY294002 (H+LY) producing a response similar to H+I with an AP accumulation ~7 fold greater than resting. Further examination showed that LY produced a biphasic response with maximal histamine enhancement at 40μM and inhibition of histamine stimulation at 80μM. This biphasic response was similar to wortmannin a PI3K inhibitor that enhances histamine stimulation at 100nM and inhibits at 10μM. Since PI3K is implicated in bulk endocytosis, relaxation of parietal cells via histamine H2 receptor antagonist (lupitidine) was tested. Secretion in response to H+LY slowed significantly after addition of lupitidine suggesting increased stimulation was not due to pumps remaining at the apical membrane, but that cells were stimulated to a higher degree. Stimulation by H+LY and H+I follow almost identical time course of AP accumulation over 30 min, whereas stimulation by H plateaus after the first 10 min. LY does not alter stimulation with carbachol or forskolin nor does it prevent inhibition caused by EGF. Thus, LY is not acting on

PI3K via the Ca2+/PKC pathway or the inhibitory EGF pathway and it must require activation of G protein-coupled H2 receptor prior to adenylate cyclase. We propose PI3K is activated during parietal cell stimulation via G protein-coupled H2 receptor and that PI3K modulates an inhibitory pathway that is overwhelmed by increased cAMP.

99

**hVps34 is a Nutrient-Regulated Lipid Kinase Required for Activation of p70 S6-Kinase by Insulin**

M. P. Byfield,[1] J. T. Murray,[2] J. M. Backer[1]; [1]Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, [2]MRC Protein Phosphorylation Unit, University of Dundee, Dundee, United Kingdom

Mammalian cells respond to nutrient deprivation by inhibiting energy consuming processes such as proliferation and protein synthesis, and by stimulating processes such as autophagy and transcriptional regulation of key metabolic genes. p70 S6-kinase (S6K1) is central to nutrient-responsive regulation of translation. S6K1 is activated by growth factors such as insulin and by mTOR, which is itself regulated by amino acids. While Class I PI 3-kinases play a well-recognized role in the regulation of S6K1, we now present evidence that the class III PI 3-kinase, hVps34, also regulates S6K1 as a critical component of the nutrient sensing apparatus. Overexpression of hVps34 or the associated hVps15 kinase activates S6K1, and insulin stimulation of S6K1 is blocked by microinjection of inhibitory anti-hVps34 antibodies, overexpression of a FYVE-domain construct that sequesters the hVps34 product PI[3]P, or siRNA-mediated knock-down of hVps34. hVps34 activity is not stimulated by insulin, and inhibition of hVps34 has no effect on phosphorylation of Akt or TSC2 in insulin-stimulated cells. This suggests that hVps34 is not part of the insulin-stimulated input to S6K1. However, hVps34 is inhibited by amino acid starvation, suggesting that it lies on the amino acid-regulated pathway to S6K1. hVps34 is also inhibited by glucose starvation and by activation of the AMP-activated Kinase (AMPK), which inhibits mTOR/S6K1 in glucose-starved cells. Our data suggest that hVps34 is a nutrient-regulated lipid kinase that integrates amino acid and glucose inputs to S6K1.

100

**Role of ZO-1 in Sphingosine-1-phosphate Receptor Regulated Endothelial Barrier Formation and Chemotaxis**

J. Lee,[1] E. Wang,[1] M. Lee[2]; [1]Gheens Center on Aging, University of Louisville Health Science Center, Louisville, KY, [2]Microbiology and Immunology, University of Louisville Health Science Center, Louisville, KY

Regulated barrier formation and chemotaxis are important functions of endothelial cells. Here, we show sphingosine-1-phosphate (S1P) by activating endothelial S1P1 receptor induce the translocation of ZO-1 to cellular migratory edges and tight-junctional structures. S1P-regulated ZO-1 translocation was inhibited by pertussis toxin, dominant-negative-Akt, -Cdc42, -Rac, -Rho, and T236A S1P1 receptor mutants, indicating S1P1-mediated signaling cascades are required. The full-length and C-terminal half of ZO-1, which contains cortactin binding site, were transfected into CHO cells and both polypeptides translocated to cellular cortical areas after S1P stimulation. In contrast, the N-terminal half of ZO-1, which fails to bind cortactin, was unable to translocate to cortical areas after S1P stimulation. Thus, the translocation of ZO-1 to cell migratory front may require cortactin association and ZO-1, a previously characterized component of tight junctions, may be critical in S1P-mediated chemotaxis. Likewise, S1P induced cortactin translocation to cellular migratory edges in a similar manner via Gi/ Akt/ Cdc42, Rho, and Rac pathway. Furthermore, double immunofluorescent staining showed both ZO-1 and cortactin were translocated to migratory edges, whereas only ZO-1 was present in endothelial tight junctions after S1P treatment. By Electric Cell-substrate Impedance Sensing technology, endothelial barrier integrity and wound-healing capability were enhanced following S1P stimulation. This enhanced barrier integrity and wound-healing capability was regulated by Gi/ Akt/ Cdc42, Rac, Rho pathway. Moreover, S1P-induced barrier integrity and wound-healing was significantly abrogated in S1P1 silenced endothelial cells. Together, these results suggest ZO-1 polypeptide may play critical roles in S1P-regulated barrier integrity and chemotaxis by the formation of two distinct ZO-1 functional complexes: ZO-1/ cortactin complexes that translocate to migratory edges to control endothelial chemotaxis and Junctional ZO-1 complexes that translocate to tight junctions to regulate endothelial barrier integrity.

101

**Small Molecule Probes of p90 Ribosomal Protein S6 Kinase Signaling**

M. S. Cohen, J. Taunton; Cellular and Molecular Pharmacology, University of California San Francisco, San Francisco, CA

p90 Ribosomal Protein S6 Kinases (RSKs) are ubiquitous downstream mediators of the Ras-mitogen-activated protein kinase (MAPK) pathway. In humans, there are four RSK isoforms (RSK1-4) of which RSK1 and RSK2 are the most extensively characterized. RSKs have been implicated in the regulation of diverse cellular processes including survival and proliferation; however, the physiological roles of RSKs remain poorly defined. Selective small molecule inhibitors of RSKs would be valuable tools to dissect their role in the MAPK pathway. We have recently described the design of a selective, irreversible small molecule inhibitor of RSK1 and RSK2 using a structural bioinformatics approach (Cohen et al. *Science* **308**, 1318). This inhibitor contains an electrophilic fluoromethylketone group that covalently modifies a non-conserved cysteine residue in the kinase active site. To explore structure-activity relationships, we synthesized a series of analogs containing various electrophilic groups. A vinyl ketone analog was identified that exhibited increased cellular potency relative to the fluoromethylketone inhibitor. We are currently using these inhibitors to probe the physiological roles of RSK in a variety of cellular contexts.

102

**The Evolutionary Origins of Phosphorylation**

Z. Serber,[1] S. M. Pearlman,[2] J. E. Ferrell[1]; [1]Molecular Pharmacology, Stanford University Medical School, Stanford, CA, [2]Computer Science, Stanford University, Stanford, CA

Life employs protein phosphorylation as a general mechanism to regulate the temporal and spatial activation of a huge variety of cellular processes. Eighteen years ago molecular biologists first realized that they can sometimes reconstitute the constitutively phosphorylated state of a protein by substituting an acidic residue, either aspartate or glutamate, for the phosphosite, a seemingly fortuitous trick that works despite differences in the side chain geometry and charge[1]. Here, we demonstrate that nature has been employing the same 'trick' for far longer, evolving some genes to encode an acidic residue, where once there was a phosphosite and vice versa. Furthermore, our analysis suggests that phosphorylation may have first evolved to reversibly mimic glutamate in crucial locations and conferred an advantage by making a protein, previously regulated only by

synthesis and destruction, into one switchable by a fast, reversible enzymatic reaction. 1. Thorsness, P. E. and Koshland, D. E., Jr.. Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. J Biol Chem 262: 10422-5.

103

**Predicted Influence of Cell Size and Shape on Global and Local Phosphorylation Levels**

D. J. Odde, J. Meyers, J. Craig; Biomedical Engineering, University of Minnesota, Minneapolis, MN

In the analysis of cell signaling the three-dimensional spatial dependence of the signal is often ignored. For example, in the case of second messengers that are generated at the plasma membrane and then diffuse in the cytoplasm to reach their targets, the second messengers are considered to have a concentration that varies only in time, and not in space. Here we use mathematical modeling of a plasma membrane-bound kinase and a cytoplasmic phosphatase to show that as a model cell grows, the substrate becomes progressively dephosphorylated due to decreased proximity of the substrate to the kinase. Conversely, as a cell flattens at constant volume, the substrate becomes phosphorylated. Also, in the leading edge of polarized cells and in protrusions such as filopodia, the substrate is highly phosphorylated relative to the bulk cytoplasm. The results demonstrate that phosphorylation can be stimulated, both globally and locally, via changes in cell size and shape. Finally, the principles apply similarly to other antagonistic systems, such as spatially segregated GEF/GAP pairs acting on G-proteins. The predicted consequence of these results is that kinase-mediated (or GEF-mediated) signaling can be turned on and off locally and globally simply by changes in cell size and shape as mediated by cytoskeletal rearrangement. The modeling predicts that cells that are well-spread on a two-dimensional surface will have a phosphorylation state different from a less-spread cell that is in a three-dimensional matrix, independent of the molar concentration of the extracellular stimulus or its downstream kinase/phosphatase and/or GEF/GAP transducers.

104

**The Role of ARNO/cytohesin-2 in $A_{2A}$-Adenosine Receptor Signaling**

I. Gsandtner, C. Charalambous, F. Eskandary, H. Farhan, M. Freissmuth, J. Zezula; Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Institute of Pharmacology, Vienna, Austria

Recent findings suggested the existence of G-protein independent signaling pathways for the $A_{2A}$-adenosine receptor. To find interaction partners necessary for signaling, we have used the intracellular carboxyl terminal tail of the $A_{2A}$-receptor as bait in a yeast-two hybrid interaction screen. One of the interacting proteins identified as ARNO/cytohesin-2. ARNO/cytohesin-2 was discovered as a nucleotide exchange factor for the small (monomeric) G proteins of the ADP-ribosylation factor (ARF) family, (while ARFs were originally identified as cofactors for cholera toxin-mediated ADP-ribosylation of $G_s$). Mutation of the $A_{2A}$-receptors C-terminal tail revealed that a minimum requirement supporting the interaction is met by the first 22 amino acids adjacent to the seventh trans-membrane region. The direct interaction found in the yeast-two hybrid system was further confirmed by mutual pull-down (of fusion proteins expressed in bacteria) and coimmunoprecipitation of the proteins expressed in mammalian cells. ARNO/cytohesin-2 had no effect on cAMP accumulation after stimulation with an $A_{2A}$-specific agonist. However, the presence of ARNO is required to support the alternative ($G_s$ -independent) signaling to mitogen-activated protein kinase, because a dominant negative version of ARNO/cytohesin-2 efficiently suppresses the sustained phase of receptor dependent MAP-kinase phosphorylation. Since treatment with Brefeldin A did not influence ERK1/2 activation we concluded that this pathway is rather dependent on Arf6 than on Arf1 or Arf3. To prove this we transfected cells with a plasmid encoding the dominant negative version of Arf6 and found an impaired second phase of ERK1/2 phosphorylation. These experiments suggest that ARNO/cytohesin-2 interacts directly with the $A_{2A}$-adenosine receptor. This interaction regulates $A_{2A}$-adenosine receptor mediated MAP-kinase via Arf6.

## Mitosis & Meiosis I (105-129)

105

**Ran Coordinates pre- and post-Metaphase Events in *Drosophila* Embryos by Regulating the Localization of Aurora A kinase and Mitotic Kinesins KLP61F and KLP3A**

R. V. Silverman-Gavrila, A. Wilde; Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada

Previously we showed that a balance of RanGDP/GTP is required for spindle assembly and function in syncytial *Drosophila* embryos. Ran GTP was required for mitotic events such as microtubule nucleation, centrosome duplication, division spindle and midbody assembly and stability, nuclear division, chromosome alignment at metaphase plate, anaphase chromosome movement, and skeletor spindle matrix organization. One molecular mechanism by which Ran regulated these mitotic events *in vivo* was the re-localization of the kinase Aurora A from centrosomes to spindle microtubules. The phenotypes induced by altered levels of RanGTP were consistent with Ran regulating the activity of motor proteins involved in spindle organization. Indeed we showed that Ran coordinates the localization of mitotic kinesins KLP61F and KLP3A. Injection of anti-Aurora A kinase antibody or Aurora A kinase inhibitors into *Drosophila* embryos caused spindle assembly and chromosome division defects that were a subset of the defects seen after perturbation of Ran pathway as well as mislocalization of KLP61F and KLP3A. Certain spindle phenotypes were seen with a higher frequency after Aurora A inhibition than after Ran pathway disruption suggesting that Aurora A activity is regulated by more than one mechanism.

106

**Analysis of a RanGTP-regulated Gradient Essential for Mitosis in Somatic Cells**

P. Kalab, A. Pralle, R. Heald, K. Weis; Molecular and Cell Biology, UC Berkeley, Berkeley, CA

The small GTPase Ran regulates interactions between nuclear transport receptors of the importin β family and cargoes. Although Ran-regulated importin β cargoes were shown to function during spindle assembly in *Xenopus* egg extracts, the role of this pathway during mitosis in somatic cells is unclear. We have examined the spatial organization of the Ran-importin β system in live cells by conventional and fluorescence lifetime microscopy (FLIM) using a model cargo, termed Rango, that increases its fluorescence resonance energy transfer (FRET) signal when released from importin β by RanGTP. While Rango release by RanGTP occurs in nuclei during interphase, Rango is predominantly free upon nuclear envelope breakdown in mitotic cells. However, the concentration of free Rango is further elevated around mitotic chromatin. *In vitro* experiments

and modeling indicate that this corresponds to a localized increase in RanGTP concentration sufficient to induce microtubule polymerization. Ablation of this cargo gradient in cells leads to defects in spindle assembly. Our analysis reveals that the Ran-importin β pathway is sensitive to small concentration differences, allowing it to locally activate essential mitotic targets in a switch-like fashion.

107

**A High-throughput Screen for Small Molecules that Disrupt the Interaction Between RanGTP and Importin-beta**

J. F. Soderholm,[1] M. Uehara-Bingen,[2] B. Wolff,[2] J. Williams,[2] K. Weis,[1] R. Heald[1]; [1]Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [2]Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA

The Ran GTPase regulates nucleocytoplasmic transport and mitotic progression through its interaction with the transport receptor importin-β. It has been hypothesized that a RanGTP gradient regulates mitotic events by releasing importin-β-bound mitotic factors in a spatially-restricted manner around the chromosomes. A direct test of this hypothesis in living cells would be feasible with a rapidly-acting small molecule inhibitor that blocks the interaction between RanGTP and importin-β. An inhibitor of this type could be used to assess the roles of proteins at specific stages of mitosis, such as spindle assembly and nuclear envelope assembly. We have designed a high-throughput, 384-well format screen to identify such compounds using a fluorescence resonance energy transfer (FRET)-based assay. Our assay includes a CFP-tagged version of Ran (the FRET donor) and a YFP-tagged version of importin-β (the FRET acceptor). When CFP-RanGTP binds to YFP-importin-β energy is transferred from CFP to YFP, leading to FRET. Our screen was designed to identify compounds that reduce FRET by disrupting the interaction between the probes. A critical component of our screen is a computer program that we wrote using Perl to manage the large volume of data generated. The program parses the data and uses it to generate color-coded “heat maps” that indicate changes in donor and acceptor emission intensities for each assay plate. The program also identifies false positives resulting from fluorescent compound interference, and generates a list of potential “real hits” that includes chemical structures. In a primary screen of 137,284 compounds, we have identified approximately 100 non-interfering compounds that interrupt the FRET signal generated by our probes. By integrating our simple fluorescence-based assay with automated data analysis, we have developed an efficient method for identifying compounds that disrupt the RanGTP/importin-β interaction.

108

**Does Nanoscale Electrostatics Move Chromosomes During Mitosis?**

L. J. Gagliardi; Physics, Rutgers University, Camden, NJ

Identifying the motive force is central to explaining chromosome motions during mitosis. Presently, there is no consensus on what it is. I have proposed a minimal assumptions comprehensive model for post-attachment chromosome motions based on nanoscale electrostatics and assumptions regarding the net charge on kinetochores and centrosomes [1,2]. The present work shows that given the electric dipole nature of tubulin and the dynamic instability of microtubules it is possible to account for prometaphase post-attachment, metaphase, and anaphase chromosome motions within a comprehensive model based on nanoscale electrostatics without any assumptions regarding the net charge of kinetochores and centrosomes. Microtubule subunits are electric dipolar structures that can act as intermediaries extending the reach of the electrostatic interaction in spite of counterion screening. This allows prometaphase post-attachment and metaphase chromosome motions to be explained by statistical fluctuations in nanoscale electrostatic antipoleward microtubule assembly forces acting at chromosome arms, kinetochores, and poles, combined with similar fluctuations in nanoscale electrostatic microtubule poleward-directed disassembly forces acting at kinetochores and spindle poles. After a bivalent attachment has been completed the "inverse square" dependence of the electrostatic antipoleward force acting at chromosome arms ensures congressional motion of chromatid pairs to the midcell region. Stable metaphase mid-cell oscillatory motion is a direct consequence of the inverse square dependence of the antipoleward forces. With the increase in free calcium concentration at the onset of anaphase, the probability for microtubule assembly is decreased significantly, allowing electrostatic microtubule disassembly forces acting at kinetochores and poles to dominate, and anaphase-A motion ensues. Anaphase-B cell elongation is addressed consistently by electrostatic repulsion between the charged free ends of polar microtubules originating from opposite poles as they disassemble. [1] L. John Gagliardi, Phys. Rev. E 66, 011901 (2002). [2] L. John Gagliardi, J. Electrostat. 63, 309-327 (2005).

109

**Biophysical Properties Governing Nuclear Shape and Size of Mitotic Fission Yeast**

G. Lim,[1] Y. Torii,[1] G. Huber,[2] J. Miller,[1] S. Sazer[1]; [1]Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, [2]Department of Cell Biology, University of Connecticut Health Center, Farmington, CT

In mitosis of the fission yeast *Schizosaccharomyces pombe*, a mitotic spindle forms and elongates within the nucleus, driving the nucleus to undergo a sequence of shape transformations (round -> oblong -> peanut -> dumbbell) before it resolves into two round daughter nuclei. The combined volume of the daughter nuclei immediately after mitosis is the same as the volume of the single nucleus at the onset of mitosis (Torii and Sazer, unpublished); consequently, the area of the nuclear envelope (NE) must increase by 26% during this process. We aim to determine the biophysical properties governing these shape and size changes. To that end, we have developed a physical model of the fission yeast nucleus. It is based on current understanding of the nuclear architecture, insight from experimental images of wild-type and mutant nuclei, and concepts from the membrane mechanics governing red blood cell shape, vesicle shape, and tube formation from bilayer membranes. The model incorporates NE bending elasticity, regulation of the NE area, nuclear lumen and nucleoplasmic volumes, and spindle elongation, as well as excluded-volume effects of the condensed chromosomes, nucleolus, and mitotic spindle. In relation to regulation of the nucleoplasmic volume, the model predicts a finite nucleoplasmic pressure. The model yields quantitative estimates (as ratios to the NE bending stiffness) of the NE membrane tension, nucleoplasmic pressure, and spindle-pushing force. More generally, the model should be useful for explaining various abnormal nuclear shapes in terms of biophysical defects.

110

**The Cell Cycle as a Finite State Machine**

J. W. Jacobberger,[1] R. M. Sramkoski,[1] D. V. Soni,[2] P. S. Frisa[1]; [1]Cancer Center, Case Western Reserve University, Cleveland, OH, [2]Environmental Health Sciences, Case Western Reserve University, Cleveland, OH

Division of centrosomes, chromatin, and the cell are morphological periodicities with sharply defined boundaries. DNA synthesis defines a biochemical periodicity. These periodicities imply underlying regulatory logic that is periodic. Regulatory biochemical activities are also periodic, however the boundaries are not usually sharply defined. Periodic synthesis and degradation of the cyclins might be described as prototype markers of biochemical periodic phenomena that are surrogates for biochemical switches. This is because (1) the activities of the cognate Cdks increase as a function of cyclin quantity; (2) different cyclins are synthesized periodically and sequentially, and (3) the periodic activities regulate down-stream cell cycle phase transitions. Pines and Rieder (Nature Cell Biol., 2001, 3:E3-E6) put forward the idea that mitosis can be described or viewed as transition states defined by levels of biochemical activities rather than morphology. They describe 5 transition states defined by dominant roles for Cyclin A/CDK, polo-like kinases, aurora B kinase; Cyclin B/Cdk1; APC-Cdc20, BubR1/Mad2; fully active APC-Cdc20; and APC-Cdh1. This constitutes a biochemical finite state model in which states are marked by specific biochemical activities. We report here that this model is supported in principal by correlated single cell measurements of some of these activities. We have measured the levels of substrates for Aurora Kinase B, Cyclin B1/Cdk1, APC/Cdc20, and fully active APC/Cdc20-APC/Cdh1 by multiparameter cytometry. The correlated measurements produce clusters that can be thought of as vectors (the data have magnitude and direction) and can be defined objectively by multi-dimensional geometric boundaries. These clusters define finite biochemical states. This approach leads to a view of the cell cycle as a biochemical finite state machine. We hypothesize that this logic can be extended from mitosis to the entire cell cycle.

111

**Ancestral Mitotic Mechanisms in the Basal Eukaryote, *Giardia lamblia***

M. S. Sagolla, S. C. Dawson, W. Z. Cande; Molecular and Cell Biology, UC Berkeley, Berkeley, CA

*Giardia lamblia*, a binucleate parasite, belongs to the earliest diverging eukaryotic lineage, the Diplomonads. *Giardia* is characterized by two diploid nuclei and a complex microtubule cytoskeleton with eight flagella. During prophase both nuclei migrate to the center of the cell while two independent bipolar spindles assemble around each nucleus. The spindles elongate to segregate daughter nuclei along the left-right axis of the cell. Transmission electron microscopy (TEM) confirmed that mitosis is semi-open, the spindle is extra-nuclear and microtubules nucleated at basal bodies enter the nucleus through openings in the nuclear membrane at each pole. The *Giardia* homolog of the centromere specific histone, CENP-A, localized to a discreet spot on each chromosome. During mitosis CENP-A localization showed a distinct anaphase a and b and chromosome segregation based on microtubule-kinetochore interactions. Treating cells with the microtubule-stabilizing drug taxol or the destabilizing drug nocodazole disrupted the integrity of the mitotic spindles and resulted in chromosome segregation defects confirming the role of microtubules in chromosome segregation. We previously showed that the flagella are present throughout mitosis and are segregated to the sides of the central mitotic nuclei. We show the conserved basal body/centriole protein centrin localized to the flagellar basal bodies during interphase and the spindle poles during mitosis, confirming our TEM images of basal bodies at the spindle poles. The localization pattern of centrin during spindle assembly is consistent with a role for flagellar basal bodies in spindle nucleation and organization. Following spindle breakdown in late telophase centrin localized to the flagellar basal bodies between the new nuclei. In order to determine the pattern of nuclear inheritance and flagellar segregation we used video microscopy of living cells to follow the migration of the nuclei and flagella during mitosis.

112

**A Mammalian Somatic Cell-Based in vitro System for Studying Mitotic Protein Turnover**

M. K. Summers, P. K. Jackson; Pathology, Stanford University, Stanford, CA

Proper execution of mitosis requires the ordered proteolysis of mitotic regulators, catalyzed by the Anaphase Promoting Complex (APC), an E3 ubiquitin ligase. Despite intense study, the mechanism(s) contributing to the timely destruction of APC substrates, for example, the differential kinetics of cyclins A and B destruction, is poorly understood. The *Xenopus* egg extract system has been a powerful tool for studying the basic mechanisms controlling ubiquitination of cyclins and other mitotic regulators by the APC and their linked destruction. However, this system does not fully recapitulate important aspects of the mitotic regulation in the somatic cell cycle, e.g. spindle checkpoint activity. Furthermore, the system is not amenable to powerful siRNA techniques and genomic data available in the human system, which together would greatly enhance our ability to identify new factors and to manipulate the system. To this end, we have established a mammalian somatic cell-based extract system for the study of mitotic destruction events. This system is competent for both ubiquitination and destruction of mitotic proteins, including cyclin A and securin. We have validated critical biochemical requirements in this system including ATP dependence (by treatment with apyrase), proteasome dependence (by treatment with MG132), and APC dependence by blocking ubiquitination with the APC inhibitor Emi1. Using this system we find that both spindle checkpoint components additional control steps at the post-ubiquitination level contribute to the timing of cyclin destruction. The results of these studies will be presented.

113

**The Mitotic Spindle Protein Astrin is a Substrate of the CDC14A Phosphatase**

M. V. Nachury,[1] B. K. Kaiser,[1] G. Mack,[2] D. Compton,[2] P. K. Jackson[1]; [1]Department of Pathology, Stanford University, Stanford, CA, [2]Department of Biochemistry, Dartmouth Medical School, Hanover, NH

In addition to being a core component of the centrosome, the human CDC14A phosphatase is a regulator of mitosis. However, very few validated substrates of CDC14A have been identified thus far. A 2-hybrid screen for partners of CDC14A identified the microtubule associated protein astrin. Since astrin is phosphorylated in mitosis and it localizes to the mitotic spindle, we set out to characterize the astrin/CDC14A interaction. First, astrin interacts with CDC14A in in vitro association assays. Secondly, CDC14A is able to partially dephosphorylate astrin. Thirdly, astrin protein levels reach a peak in mitosis and rapidly decline after mitotic exit in a proteasome-dependent manner. Finally, depletion of astrin from cultured cells results in a significant mitotic exit delay. We are currently following preliminary results showing that astrin associates with the dynein/dynactin complex in a phosphorylation-dependent manner. Together with recent identification of MKLP1 as a substrate of CDC14A, this study will shed light on the regulation of mitosis by the CDC14A phosphatase.

114

**Efficient Mitosis in Human Cells Lacking Poleward Microtubule Flux**

N. J. Ganem, K. S. Upton, D. A. Compton; Biochemistry, Dartmouth Medical School, Hanover, NH

Chromosome segregation relies on the dynamic properties of spindle microtubules (MTs). Poleward MT flux contributes to spindle microtubule dynamics through the disassembly of microtubule minus ends at spindle poles coupled to the continuous poleward transport of spindle microtubules. Despite being conserved in metazoan cells, the function of flux remains controversial because flux rates differ widely in different cell types. For example, in meiotic systems, flux is relatively fast and nearly equivalent to the rate of chromosome movement. In contrast, flux is relatively slow in mitotic systems and significantly slower than chromosome movement. Here, we show that human mitotic cells depleted of the kinesin-13 proteins Kif2a and MCAK display high frequencies of lagging chromatids at anaphase and the spindles lack detectable flux. Elimination of flux reduces poleward chromosome velocity ~20%, but does not hinder bipolar spindle assembly, chromosome alignment or mitotic progression. These data demonstrate that mitosis proceeds efficiently in human cells without detectable poleward microtubule flux, and that kinetochores are sufficient to effectively power chromosome movement. We speculate that the selective pressure to maintain flux in cells where it contributes little to chromosome movement derives from the role it plays in regulating kinetochore activity and/or providing plasticity to microtubule attachment at kinetochores and spindle poles.

115

**Chemical Biology of Cell Division: New Probes for Monopolar Mitoses**

U. Peters, J. Cherian, J. H. Kim, B. H. Kwok, T. M. Kapoor; Chemistry and Cell Biology, Rockefeller University, New York, NY

To identify small molecules that affect a cellular process in different ways, it is not known whether a screen with unrelated compounds spanning a vast region of chemical space, or with compounds related to a 'privileged scaffold' focused on a small region of chemical space, is needed. To test the latter possibility, we synthesized a collection of ~100 compounds based on a diaminopyrimidine scaffold, a motif common in ATPase and kinase inhibitors. We carried out a microscopy-based screen examining the dose-dependent effects of these compounds on mammalian mitosis. Monopolar and multipolar spindles, bipolar spindles with disorganized microtubules or unaligned chromosomes, and chromosome segregation defects were observed. The number of phenotypes in this chemical screen was comparable to that observed in RNAi-based genome-wide screens. Our studies on the mechanism of action of these compounds started with those that resulted in monopolar mitoses. In vitro assays with recombinant Eg5, a kinesin required for bipolar spindle assembly, excluded it as a direct target of these compounds. Analyses of RNAi-phenotypes for mitotic kinases suggested that polo-like kinase 1 (Plk1) may be a possible target. Kinase assays confirmed that one compound, named DAP-81, inhibits Plk1 (IC50~1μM). In another recent study, compounds closely related to DAP-81 have also been reported to inhibit Plk1 in vitro. We show that DAP-81 inhibits the phosphorylation of Plk1 substrates in cells at concentrations where the phosphorylation of aurora kinase substrates was unchanged. As normal bipolar spindles do not form after Plk1 RNAi, DAP-81 provided a tool to examine Plk1 function in assembled bipolar spindles. We find that Plk1 contributes to chromosome-microtubule interactions and to the maintenance of normal bipolar spindles. Structurally unrelated chemical inhibitors of Plk1 were used in parallel experiments to confirm the results obtained with DAP-81.

116

**A Catalytic Subunit of Phosphatase 2A(PP2A) Modulates Pulling Forces During Asymmetric Division of One-cell Stage *C elegans* Embryos**

K. Afshar, P. Gönczy; ISREC/EPFL, Epalinges, Switzerland

In one-cell stage *C. elegans* embryos, asymmetric positioning of spindle is achieved by an imbalance of pulling forces on spindle poles, with more force pulling on the posterior spindle pole (Grill et al, 2002). Heterotrimeric G proteins are essential for this process, as simultaneous inactivation of two Galpha subunits, GOA-1 and GPA-16, as well as that of their positive regulator GPR-1/2, results in extreme reduction of pulling forces and symmetric division (Colombo et al, 2003). How these proteins are modulated to ensure accurate force generation remains incompletely understood. We identified a catalytic subunit of PP2A (hereafter refered to as PP2A) as a novel modulator of pulling forces. Inactivation of PP2A results in reduced pulling forces on both spindle poles. Nevertheless, imbalance of pulling forces is retained and the first division is asymmetric. Immunolocalization studies reveal that PP2A is mainly cytoplasmic but also localizes to centrosomes. We found that PP2A physically interacts with GPR-1/2 in yeast two hybrid assay and in co-immunoprecipitation experiments. In addition, we found that PP2A is required for efficient interaction of GPR-1/2 with both GOA-1 and GPA-16. Accordingly, the phenotypes of *goa-1* and *gpa-16* mutant embryos are enhanced upon inactivation of PP2A in addition. Importantly, we detected two GPR-1/2 species by 2D gel analysis, one of which is diminished relative to the other upon inactivation of PP2A. Together, our results indicate that dephosphorylation of GPR-1/2 by PP2A is necessary for accurate generation of pulling forces during asymmetric spindle positioning.

117

**The Role of Vertebrate Cohesin in Mitotic Spindle Formation**

X. Kong,[1] E. Sonoda,[2] S. Takeda,[2] K. Yokomori[1]; [1]Biological Chemistry, University of California, Irvine, Irvine, CA, [2]Kyoto University, Kyoto, Japan

Cohesin is an essential protein complex required for sister chromatid cohesion. Cohesin associates with chromosomes and establishes sister chromatid cohesion during interphase. During metaphase, only a small amount of cohesin remains at the chromosome-pairing domain, while the majority of cohesin resides in the cytoplasm. The significance of the cytoplasmic cohesin was unclear. We describe the mitosis-specific recruitment of cohesin to the spindle poles through its association with centrosomes and interaction with NuMA. Cohesin depletion abolished mitotic spindle aster assembly in vitro, which was rescued by mitotic, but not S phase nuclear, cohesin, indicating the mitosis-specific function of cohesin in spindle assembly. Depletion of cohesin using siRNAs in human cells and conditional knockout in chicken DT40 cells resulted in abnormal mitotic spindle assembly in vivo. These results identify a novel spindle-associated role for vertebrate cohesin during mitosis, in addition to its function in sister chromatid cohesion.

118

**Development of X tropicalis Egg Extracts as a Model System: Insights into Spindle Length Regulation**

T. J. Maresca, M. D. Blower, K. S. Brown, T. C. Grammer, R. M. Harland, R. Heald; Molecular and Cell Biology, UC-Berkeley, Berkeley, CA

The biochemical and cytological accessibility of the *Xenopus laevis* egg extract system has been instrumental in studies of fundamental cellular processes occurring throughout the cell cycle, including DNA replication, nucleocytoplasmic transport, chromosome condensation and spindle assembly. However, a deficiency of sequence data and its pseudotetraploid genome has limited the use of *X. laevis* for genetic and proteomic approaches. The related frog *Xenopus tropicalis* has emerged as an organism that expands the range of possible experiments. *X. tropicalis* are smaller, diploid, and have a shorter generation time, making them more amenable to genetics. Extensive genome sequence data are available for *X. tropicalis*, facilitating rapid protein identification by mass spectrometry, and the development of specific chromosome probes. To test whether the smaller *X. tropicalis* eggs could also be utilized for in vitro experiments, we prepared cytoplasmic extracts and tested them in several assays. As for *X. laevis*, *X. tropicalis* extracts could induce *X. laevis* sperm nuclei to transit through the cell cycle, inducing nuclear envelope assembly during interphase, and spindle assembly during metaphase. *X. laevis* antibodies revealed similar staining patterns on *X. tropicalis* spindles by immunofluorescence, and recognized homologous proteins as determined by Western blot or immunoprecipitation and mass spectrometry analysis. Whereas *X. laevis* extracts contained multiple variants of most proteins, all *X. tropicalis* extracts proteins examined existed as single isoforms, reflecting the difference in ploidy of the two species. Interestingly, *X. tropicalis* spindles were ~30% smaller than *X. laevis* spindles. We are currently investigating how cytoplasmic activities contained within each extract define spindle length. Thus, in addition expanding the possible range of experiments, the use of *X. tropicalis* may provide novel insight into spindle morphogenesis.

119

**Three Human Kinesin-8 Family Members, Kif18A, Kif18bB and Kif19, Perform Distinct Roles in Mitosis**

T. M. Sonbuchner, D. W. Buster, D. J. Sharp; Physiology and Biophysics, Albert Einstein College of Medecine, Bronx, NY

Kinesins are mechanochemical proteins that use ATP to power multiple cellular processes (including transport and mitosis) while binding microtubules (MT). Recently Kinesin-13's (kinesins with internal motor domains) have received notice because of their seemingly unique ability to depolymerize MTs. However, another class of kinesins, termed Kinesin-8s, has been found to have this same property. Using a human osteosarcoma cell line (U2OS) we have cloned the three human Kin-8's, termed Kif18A, Kif18B, and Kif19. Using siRNA to suppress the expression of each, we have found that all three significantly impact mitosis. Interestingly, real time imaging indicates distinct impacts on spindle and chromosome dynamics. Thus, humans have three Kinesin-8 family members with important and distinct roles in mitosis.

120

**Distinct Roles for Three Kinesin-13 Proteins During Mitosis in Human Cells**

N. J. Ganem, D. A. Compton; Biochemistry, Dartmouth Medical School, Hanover, NH

Kinesin-13 family members are characterized as having an internal catalytic motor domain known to induce microtubule depolymerization. The human genome encodes three distinct Kinesin-13 family members, Kif2a, Kif2b, and MCAK (Kif2c). MCAK localizes to kinetochores and spindle poles and is essential for correction of improper attachment of microtubules to kinetochores. MCAK deficiency does not alter bipolar spindle assembly or chromosome movement, but increases the frequency of lagging chromatids at anaphase. Kif2a is essential for bipolar spindle assembly. It localizes predominantly to spindle poles where it induces disassembly of microtubule minus ends needed for poleward microtubule flux. Here, we examine the mitotic role of Kif2b, the third member of the Kinesin-13 family in humans. Following knock-down of Kif2b using siRNA, we observe that a majority of cells assemble abnormal spindles with highly disorganized microtubules. Chromosomes in these cells do not display typical oscillatory motion and the velocity of movement is severely depressed. In the minority of cells that build bipolar spindles following Kif2b siRNA treatment, chromosomes align and segregate at anaphase, but cytokinesis fails and the cleavage furrows regress leading to binucleate cells. These data demonstrate that Kif2b is important for mitotic spindle assembly, chromosome movement, and completion of cytokinesis, and indicate that each of the Kinesin-13 family members play functionally distinct roles during mitosis in human cells.

121

**Myosin Phosphatase Targeting Subunit1 Controls Sister Chromatid Segregation by Phosphorylation-dependent Association with Polo-like Kinase1**

S. Yamashiro,[1] Y. Yamakita,[1] G. Totsukawa,[1] H. Goto,[1] K. Kaibuchi,[2] M. Ito,[3] D. Hartshorne,[4] F. Matsumura[1]; [1]Mol. Biol. & Biochem., Rutgers, Piscataway, NJ, [2]Cell Pharmacology, Nagoya University, Nagoya, Japan, [3]Internal Medicine, Mie University, Tsu, Japan, [4]Nutritional Sciences, University of Arizona, Tucson, AZ

The protein phosphatase 1 (PP1C) catalytic subunit forms a complex with a variety of regulatory molecules (phosphatase targeting subunits), which converts PP1C into many forms of phosphatase with distinct substrate specificities. One of such targeting molecules is the myosin phosphatase targeting subunit1 (MYPT1). MYPT1, when complexed with PP1C, can effectively dephosphorylate several substrates including myosin light chain, moesin and adducin, and, therefore is believed to control microfilament organization. Surprisingly, we have found that MYPT1 controls sister chromatid segregation by its association with polo-like kinase1 (PLK1), the molecule of which is known to regulate a variety of mitotic events including chromosome segregation. MYPT1 was phosphorylated by cdc2 kinase in a mitosis-specific way, and the phosphorylation sites are well conserved among vertebrate MYPT1. Interestingly, one (Ser473) of these sites constitutes a motif (Ser-phosphoSer-Pro) for phosphorylation-dependent binding to the polo-box domain of PLK1. Indeed, MYPT1 and PLK1 co-immunoprecipitated as a complex. GST pull-down assays confirmed that MYPT1, when phosphorylated by cdc2, directly bound to PLK1. MYPT1 depletion by siRNA in HeLa and SW962 cells resulted in mitotic arrest. Giemsa staining of mitotic chromosome spreads demonstrated that cohesion of sister chromatids at the centromere was disrupted in MYPT1-depleted cells whereas control chromosome retained tight association of sister chromatids at the centromere. An antibody specific to Ser473-phosphorylated MYPT1 revealed that phosphorylated MYPT1 co-localized with PLK1 at the kinetochores. Depletion of PLK1 disrupts the kinetochore localization of MYPT1, indicating that MYPT1's localization at the kinetochore depends on PLK1. PLK1 is reported to facilitate chromosome segregation by phosphorylating components of the cohesin complex. Our results thus suggest that the MYPT1/PP1C complex is a phosphatase that counteracts PLK1, preventing premature sister chromatid segregation before metaphase-anaphase transition.

122

**The C elegans Tousled-like Kinase TLK-1 Contributes to Mitotic Chromosome Segregation as a Substrate and Regulator of the Aurora B Kinase AIR-2**

Z. Han,[1] G. M. Riefler,[1] J. R. Saam,[2] S. E. Mango,[2] J. M. Schumacher[1]; [1]Molecular Genetics, University of Texas MD Anderson Cancer Center, Houston, TX, [2]University of Utah Huntsman Cancer Institute, Salt Lake City, UT

The Aurora kinases control multiple mitotic processes including centrosome maturation, spindle assembly, chromosome segregation, and cytokinesis. Aurora activity is regulated in part by a subset of Aurora substrates that once phosphorylated, can enhance Aurora kinase activity. Aurora A substrate activators include TPX2 and Ajuba, while the only known Aurora B substrate activator is the chromosomal passenger INCENP. We report that the C. elegans Tousled kinase TLK-1 is a second substrate activator of the Aurora B kinase AIR-2. Tousled kinase (Tlk) expression and activity have been linked to ongoing DNA replication and Tlk can phosphorylate the chromatin assembly factor Asf. We found that TLK-1 is phosphorylated by AIR-2 during prophase/prometaphase, and that phosphorylation increases TLK-1 kinase activity in vitro. Phosphorylated TLK-1 increases AIR-2 kinase activity in a manner that is independent of TLK-1 kinase activity but depends on the presence of ICP-1/INCENP. In vivo, TLK-1 and AIR-2 genetically cooperate to ensure proper mitotic chromosome segregation. These results suggest that Tousled kinases have a previously unrecognized role in mitosis and that Aurora B associates with discrete regulatory complexes that may impart distinct substrate specificities and functions to the Aurora B kinase.

123

**TD-60, a Putative Guanine Exchange Factor, Regulates Chromosome Congression and Cytokinesis by Controlling Aurora B Kinase Activity**

S. E. Rosasco, W. Lan, P. T. Stukenberg; Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA

Aurora B is a mitotic serine/threonine kinase involved in the regulation of kinetochore-microtubule attachment and the positioning of the cytokinetic furrow. It exists in a complex with the passenger proteins INCENP, Survivin and Borealin/Dasra during mitosis. TD-60 is a putative guanine exchange factor (GEF) that has also been identified as a mitotic passenger protein. We have shown that embryos injected with TD-60 antibodies exhibit a failure to undergo cytokinesis, and extracts depleted of TD-60 fail to align chromosomes; two phenotypes consistent with misregulation of Aurora B kinase activity. TD-60 physically interacts with a select population of the Aurora B complex in *Xenopus* embryonic extracts. In both extracts and in vitro, TD-60 regulates Aurora B kinase activity by controlling phosphorylation on the key regulatory residue S850 of INCENP by Aurora B. TD-60 also interacts with PP1 and controls the interaction of PP1 with the Aurora B complex. We propose that TD-60 regulates PP1 to modulate Aurora B kinase activity during mitosis.

124

**Inhibition of Polo-like Kinase 1 by Antibody Microinjection Inhibits the Mitotic Spindle Checkpoint and Causes Aberrant Mitotic Exit**

J. R. Daum,[1] W. Lan,[2] P. Stukenberg,[2] G. J. Gorbsky[1]; [1]Molecular, Cell, and Developmental Biology, OMRF, Oklahoma City, OK, [2]Department of Biochemistry and Molecular Genetics, University of Virginia Medical School, Charlottesville, VA

Polo-like kinase-1 (Plk1) is an evolutionarily conserved serine/threonine protein kinase. Its activities influence the assembly and function of the mitotic spindle, mitotic checkpoint signaling, chromosome segregation, and cytokinesis. We have previously demonstrated that Plk1 is the kinase responsible for creation of the tension-sensing 3F3/2 kinetochore phosphoepitope and that it modulates the association of certain mitotic checkpoint proteins with kinetochores of mitotic chromosomes. We have also shown that Plk1 accumulation at kinetochores and perhaps its kinase activity are regulated by microtubule attachment to and tension at kinetochores. We now show that inhibition of Plk1 by antibody microinjection causes mitotic cells to exit mitosis prematurely without separating sister chromatids or completing cytokinesis. Moreover, microinjection of anti-Plk1 antibody overrides the spindle checkpoint in cells arrested by treatment with microtubule poison. In contrast, cells arrested at the metaphase-to-anaphase transition by the addition of the 26S proteosome inhibitor, MG132, do not exit mitosis after injection of anti-Plk1 antibody. Thus, proteasome-mediated proteolysis is required for mitotic exit triggered by inhibition of Plk1. The activity of Aurora B, another kinase required for the maintenance of the mitotic checkpoint, is not inhibited by injection of anti-Plk1 antibody. However, inhibition of Aurora B removes both the 3F3/2 phosphoepitope and Plk-1 from kinetochores. These data suggest that Plk1's role in the regulation of the mitotic spindle checkpoint is downstream of Aurora B. Consequently the failure of cells to maintain mitotic arrest after Aurora B inhibition may be due to a subsequent inhibition of Plk1.

125

**Mechanism of Chromosome Positioning on the Spindle Equator in *Drosophila***

H. Maiato,[1] A. J. Pereira,[1] A. Khodjakov,[2] C. L. Rieder[2]; [1]Inst. for Mol. and Cell Biol., Univ. of Porto, Porto, Portugal, [2]Molecular Medicine, Wadsworth Center, Albany, NY

Fully-congressed metaphase chromosomes in *Drosophila* S2 cells remain positioned at the spindle equator after severing one of the two kinetochore fibers (K-fibers) with a laser microbeam (Maiato et al., 2004, JCB, **167**:831-840). Surprisingly, chromosome position is not affected even when one of the K-fibers is cut <1 μm away from the kinetochore, while the other fiber is ~5 μm long. To determine if the kinetochore itself is required to 'anchor' a chromosome at the equator we ablated single kinetochores on metaphase chromosomes in cells stably expressing GFP-Cid/CENP-A (a kinetochore marker). Under this condition chromosomes consistently moved towards the pole to which the remaining kinetochore was attached. Similarly, when metaphase chromosomes were severed through the centromere, between the sister kinetochores, both fragments (each containing a single kinetochore) moved poleward. This movement was not driven by microtubule flux or cytoplasmic dynein, since it was not inhibited in cells treated with nanomolar concentrations of taxol or after depleting dynein heavy chain by RNAi. Finally, when we cut through the arms of metaphase chromosomes tagged with GFP-Histone H2B, the position of the kinetochore-containing chromosome fragment remained unchanged, while acentric fragments moved poleward at a highly variable rate. Thus, the distribution of forces acting on chromosome arms in S2 cells differs from that in vertebrates. Our results reveal that a balance of forces acting on the two sister kinetochores is required to maintain the position of a congressed chromosome at the spindle equator. Further, these forces are not proportional to the length of the K-fibers attached to the kinetochores.

126

**Asator, A Novel Tau-tubulin Kinase Interacts With the Spindle Matrix Complex During Mitosis in *Drosophila***

H. Qi, Y. Ding, L. Zhu, M. J. Blacketer, J. Girton, K. M. Johansen, J. Johansen; Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA

In *Drosophila* we have recently identified three nuclear proteins, Skeletor, Chromator, and Megator that redistribute during prophase forming a fusiform spindle structure that persists in the absence of polymerized tubulin. The features of this structure are compatible with those of a spindle matrix which on theoretical grounds has been proposed to provide a stationary substrate that anchors molecules during force production and microtubule sliding. Two of these proteins, Skeletor and Chromator, are localized to chromosomes during interphase whereas the third, Megator, occupies the intranuclear space surrounding the chromosomes. Megator is a 260 kD protein with a large $NH_2$-terminal coiled-coil domain which we here show interacts with EAST, another large protein in *Drosophila* that associates with a nuclear nonchromosomal compartment. Furthermore, by yeast two-hybrid interaction assays we have identified a novel tau-tubulin kinase (CG11533) that we have named Asator, which interacts with the $NH_2$-terminal coiled-coil domain of Megator. The kinase domain of Asator is highly conserved and in *Drosophila* alternative splicing gives rise to three isoforms with different $NH_2$-terminal domains. By P-element excision we generated a likely null allele of Asator which eliminates all three isoforms and is homozygous lethal. Transient expression studies in S2 cells revealed that Asator localizes to the cytoplasm at interphase but redistributes to the spindle matrix during mitosis. Thus, these findings indicate that EAST and Asator are likely to be the fourth and fifth members of a group of proteins derived from three different cellular compartments that reorganize during mitosis to interact within a spindle matrix complex. Experiments are in progress to determine how the reorganization of these proteins is coordinated and how the resulting molecular complex affects chromosome dynamics and cell division. (Supported by NSF grant MCB0445182).

127

**The Roles of the Microtubule-Severing Enzymes, Spastin, Fidgetin, and Katanin, in Mitotic Spindle and Chromosome Dynamics**

D. Zhang,[1] G. C. Rogers,[2] D. Buster,[1] D. J. Sharp[1]; [1]Department of Physiology&Biophysics, Albert Einstein College of Medicine, Bronx, NY, [2]Department of Biology, University of North Carolina, Chapel Hill, NC

The poleward flux of tubulin within kinetochore-associated microtubules is important for normal chromatid-to-pole motion during anaphase. This poleward flux requires the active depolymerization of microtubule minus-ends by kinesin-13s and it has been hypothesized that microtubules must be severed from a stabilizing cap on their minus-ends for this to occur. Three members of the AAA (ATPase associated with diverse cellular activities) family of proteins, Spastin, Katanin and Fidgetin have characteristics that make them likely candidates to carry this out. To examine which if any of these proteins function in such a manner, we systematically depleted each (individually and in combination) from Drosophila S2 cells using RNAi. The resulting phenotypes were assessed using 4-D spinning disk confocal microscopy of live mitotic cells stably expressing EGFP-tubulin. Although katanin has long been considered to be the primary microtubule severing enzyme in mitotic cytoplasm, our findings suggest that Spastin and Fidgetin play more prominent roles in mitotic spindle and chromosome dynamics. Specifically, the depletion of Spastin or Fidgetin significantly reduced the velocity of poleward chromatid motility relative to control treated cells ($4.90\times10^{-3}$ or $4.49\times10^{-3}$ vs $7.41\times10^{-3}$ µm/sec, respectively). Alternatively, Katanin depletion resulted in only a moderate and statistically insignificant reduction in poleward chromatid velocities. Analyses of poleward flux and in vivo protein dynamics are currently underway.

128

**Spindle Breakdown is Coordinated by a Global Disassembly Signal in Budding Yeast**

J. N. Molk, E. D. Salmon, K. Bloom; Biology, University of North Carolina, Chapel Hill, NC

Anaphase B in eukaryotes separates the genome prior to the activation of cytokinesis. If the central spindle disassembles prematurely cell viability may be compromised. Spindle breakdown occurs at the end of anaphase when microtubules in the central spindle synchronously disassemble. We have investigated the signal for spindle disassembly in budding yeast using heterokaryons where two spindles form in the same cell. This signal could be global or local. Anaphase onset began asynchronously in cells with two spindles. In both *kar9Δ* and *bik1Δ* cells the spindle that elongated first was often segregated into the daughter and began anaphase approximately eight minutes before the spindle retained in the mother cell (n = 25/37 cells). This supports a local signal for anaphase onset in the nucleus. Preanaphase spindles that repeatedly passed the bud neck displayed a delay in anaphase onset before elongating into the daughter suggesting the local signal responds to movement past the bud neck. However, both spindles broke down within approximately three minutes of each other (n = 28 cells) suggesting spindle disassembly is regulated globally. Spindle breakdown could be delayed if the spindle that elongated into the daughter cell was completely pulled into the bud prior to anaphase onset. These results suggest spindle disassembly occurs via a global signal in budding yeast and this signal is inhibited if the entire spindle enters the bud.

129

**Molecular Pathways Ensuring the Coordination of Late Cytokinetic Events with Chromosome Segregation**

Y. P. Barral, C. Norden, J. Dobbelaere, M. Mendoza; Biology, Swiss Federal Institute of Technology, Zürich, Switzerland

During anaphase, spindle elongation and shrinkage of kinetochore microtubules pull sister chromatids apart, while cytokinesis cleaves the cell between the two separated copies of the genome. How the cell ensures that spindle elongation fully separates sister chromatids along their entire length and clears the cleavage plane of chromatin is still unknown. Indeed, to fulfill this requirement the spindle must become more than twice as long than the longest chromosome arm, and spindle breakdown and the completion of cytokinesis must be delayed until that length is reached. We use budding yeast as a model to investigate the mechanisms linking chromosome separation and cell cleavage. Here we report on two opposite signaling pathways, NoCut, and Reversion-of-NoCut, that fine-tunes the timing of abscission relative to chromosome segregation. Abscission is the last and only irreversible event of cytokinesis. NoCut depends on the integrity of the spindle midzone, the aurora kinase Ipl1, the anillin-related proteins Boi1 and Boi2, and represses abscission during anaphase and in response to chromosome separation defects. Inactivation of this pathway increases the frequency of chromosome breakage by the cleavage machinery in anaphase compared to wild type. Furthermore, simultaneous inactivation of NoCut and impairment of spindle elongation causes extensive chromosome breakage and cell death. In these cells, impairment of cytokinesis suppresses chromosome breakage, demonstrating that NoCut prevents chromosome breakage by the cleavage machinery. Reversion-of-NoCut depends on centromeric chromatin, proper kinetochore assembly and PP2A activity, and upon proper chromosome separation it is required

to revert the NoCut-dependent inhibition of abscission. Thus, together our results suggest that a sensing mechanism monitors chromosome separation and/or the presence of chromatin in the midzone area to ensure that cytokinesis is completed only after that the spindle has reached the right size.

## G1-S/DNA Replication (130-142)

130

### Cell Cycle Dynamics Analysis of Chromatin Proteome in Xenopus Egg Extract

G. A. Khoudoli,[1] I. M. Porter,[1] G. X. Stewart,[1] J. S. Andersen,[2] J. R. Swedlow,[1] J. Blow[1]; [1]Division of Gene Regulation and Expression, University of Dundee, Dundee, United Kingdom, [2]University of Southern Denmark, Odense, Denmark

The Xenopus cell free egg extract recapitulates most events of early embryonic cell cycles on added demembranated sperm nuclei. We have employed this system for proteomic analysis of mitotic chromosomes and interphase chromatin at different stages of DNA replication. Reconstituted mitotic chromosomes or chromatin progressing through S-phase in the presence or absence of various replication inhibitors were isolated and proteins bound to DNA were eluted and subjected to proteomic analysis. We optimized sample preparation for 2D gel electrophoresis using Taguchi method (Khoudoli et al., Proteome Sci. 2004, 2:6). The resulting protocol, substantially improved the solubility and resolution of protein mixtures derived from a variety of sources on 2DE. Using 2DE and LC/MS/MS, we have identified 50 candidate polypeptides that associate with chromatin in a cell cycle- and replication-dependent manner and 9 uncharacterized proteins on mitotic chromosomes. Known components of replication machinery with expected behaviour, such as ORC and Mcm proteins, were among identified the polypeptides. An analysis of individual spots by immunoblotting and mass spectrometry revealed previously uncharacterized posttranscriptional modifications of Mcm(2-7). To assess the functional role of candidate proteins we raised antibodies against 9 polypeptides that were not previously associated with replication. An immunocytochemical analysis of replicating nuclei in Xenopus egg extract demonstrated that 7 of 9 antibodies recognised epitopes only in interphase nuclei and showed no staining on metaphase chromosomes. We are currently using these antibodies to verify the response of the proteins to cell cycle progression and to conduct functional studies. Our preliminary data shows that two candidate proteins participate in a functional complex. siRNA approach for the functional analysis was established and validated in study of novel proteins identified to be associated with mitotic chromosomes reconstituted in Xenopus egg extract.

131

### The Function of Mus81 in the Absence of Sgs1 in *Saccharomyces cerevisiae*-Study of Mus81 Ts$^-$mutants

M. Ii, T. Ii, S. J. Brill; Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ

Mus81-Mms4 is a conserved structure-specific endonuclease. In vitro, the budding yeast enzyme cleaves 3' flaps from a variety of branched DNA substrates. Defects in *MUS81* or *MMS4* result in strong CPT and MMS sensitivity, weak UV sensitivity, and sporulation defects, suggesting Mus81-Mms4 functions in DNA repair and meiosis. *MUS81* and *MMS4* are essential in the absence of *SGS1* or *TOP3* that encode DNA helicase and DNA topoisomerase, respectively. This suggests that Mus81-Mms4 shares overlapping function with Sgs1-Top3. The synthetic-lethality of *mus81 sgs1* is suppressed by deletion of *RAD51*. Rad51 is a component of homologous recombination (HR) machinery. Therefore, Mus81-Mms4 has been thought to function in the HR pathway like Sgs1-Top3. However, we found that *MUS81* functions primarily in a *RAD51*-independent manner and that only *SGS1* acts downstream of *RAD51*. This suggests that Mus81-Mms4 may not function in the HR pathway. To understand what function Mus81-Mms4 has in the absence of Sgs1-Top3, we carried out a screen of Mus81 Ts$^-$ mutants. PCR-mutagenized *MUS81* was transformed into a *mus81 sgs1* strain carrying wt *SGS1* on a *URA3 CEN* plasmid together with linearized *TRP1 CEN* plasmid harboring homologous regions of *MUS81*. The transformants were subjected to 5-FOA selection and then the viable cells were transferred to YPD plates and examined temperature-sensitivity. The candidates were confirmed if the Ts$^-$ phenotype depends on the *TRP1* plasmid. The Ts$^-$ mutants obtained show severe defects in cell cycle progression in *sgs1* background. Pulsed-field gel analysis revealed that the chromatin structure of Mus81 Ts$^-$ mutants in *sgs1* background changed at non-permissive temperature. Moreover, morphology of Mus81 Ts$^-$ mutants in *sgs1* background changed dramatically after shifting to 37°C. We will present further analysis of the mutant phenotype and discuss the results.

132

### Mutation Rate Analysis in Novel *Schizosacchromyces pombe* replication initiation defective (*rid*) strains

J. M. Sierra-Montes, G. D'Urso; Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL

We have recently discovered a novel checkpoint phenotype associated with mutants defective in DNA replication initiation called *rid* (for replication initiation defective). When grown under semi-permissive conditions, *rid* mutants display a cell cycle delay that is *chk1*-dependent, *cds1*-independent suggesting that a novel checkpoint pathway is activated. By analyzing mutation rates in the presence or absence of *chk1*, we have determined whether the checkpoint plays a role in either the stimulation or suppression of a mutator phenotype. For each *rid* mutant, mutation rates were also measured in the absence of *cds1+* or in the absence of both *chk1+* and *cds1+*. The forward mutation rate assays using 5-fluoroorotic acid (FOA), which detects mutations inactivating the *ura4+* gene, and canavanine, which selects against *CAN1+*, were used for the identification of the mutator phenotype. Interestingly, we have found that *rid* mutants, in contrast to mutants that delay S phase progression, do not display a mutator phenotype suggesting that blocks to initiation may be less mutagenic than blocking chain elongation. The data shows that no *rid* strain at their respective *rid*-activation temperature displays a mutator phenotype in the presence of both *chk1+* and *cds1+*. However, in the absence of *chk1+*, some of the *rid* strains (*i.e* cdc20-M10) display an increase in mutation rate, consistent with *Chk1+* having a role in preventing accumulation of DNA damage when replication initiation is delayed. As expected, no such increase in mutation rates was observed in the absence of *cds1+*. Therefore, we conclude that *chk1+* is required during the cell cycle, either at the G1/S phase transition, or later in G2, to protect cells from DNA damage caused by blocks to DNA replication initiation.

133

### Chromatin Modification Regulates Rad9 Function in DNA Damage Checkpoint Response

R. Wysocki,[1] A. Javaheri,[1] S. Allard,[2] J. Cote,[2] S. J. Kron[1]; [1]Center for Molecular Oncology, University of Chicago, Chicago, IL, [2]Laval University

Cancer Research Center, Hotel-Dieu de Quebec (CHUQ), Quebec City, PQ, Canada
The prevailing model for DNA damage checkpoints is based on studies in budding yeast that suggest single strand DNA formed via 5'-3' resection at double strand breaks (DSBs) is both necessary and sufficient for checkpoint arrest. However, the megabase domains of phosphorylated histone H2AX formed rapidly at DSBs in metazoans recruit BRCA1, 53BP1, MDC1 and other checkpoint signaling proteins, and recent data implicate chromatin regulators in yeast DNA damage response. The relative significance of DNA damage processing and/or chromatin modification in determining checkpoint response remains poorly defined. Using the yeast G1 checkpoint response to DSBs as a model, we have established essential roles for chromatin modification in DNA damage signaling and cell cycle arrest. A genomic screen for G1/S checkpoint defects after ionizing radiation revealed the conventional checkpoint regulators but also the Dot1 histone H3 Lys79 methylase. Molecular analysis demonstrated that activation and function of the 53BP1 homolog Rad9 requires assembly onto chromatin adjacent to DSBs. This assembly is mediated by binding between Rad9 tudor domains and H3 methyl-Lys79. Mutants failed to activate Rad9 or Rad53 or perform G1 arrest. Defects in the Tel1/Mec1 dependent phosphorylation of histone H2A Ser129 also attenuate Rad9 function. By contrast, loss of NuA4-dependent acetylation of histone H4 amino-terminal tails promotes Rad9 signaling and delays checkpoint recovery. These data suggest 1) a histone code for DNA damage response, mediated by both constitutive and regulated modifications and 2) a role for DSBs in assembling a chromatin domain required for DNA damage checkpoint signaling. Insofar as 5' resection of DSBs may be blocked in G1, thereby eliminating ssDNA as a potential signal, we hypothesize that assembly of Rad9 onto chromatin at sites of DNA damage may be both necessary and sufficient for yeast G1 checkpoint arrest.

134

**Population Polymorphisms in *Sciara* DNA Puff II/9A Reveal Conservation of Origin *Cis*-elements**

E. A. Gustafson, Y. Yamamoto, H. Smith, S. A. Gerbi; Mol Bio, Cell Bio, Biochem., Brown Univ. BioMed Div., Providence, RI

DNA puffs of the larval salivary gland polytene chromosomes of the fly *Sciara coprophila* exhibit developmentally regulated tissue-specific DNA amplification. We have sequenced an 8.9 kb sequence from DNA puff II/9A. It initiates replication from a small 1 kb origin region during amplification. The top strand transition point (TP1) between leading and lagging strand synthesis has been mapped to a single nucleotide within the 1 kb origin. A DNase hypersensitivity site (DHS) is found upstream of TP1 and a region of bent DNA is located between the DHS and TP1. Downstream of the origin are II/9A genes 1 and 2. Putative ecdysone response elements (EcREs) are present in the origin and promoter regions. PCR of the genomic *Sciara* II/9A locus revealed a series of multiple-sized PCR products. Cloning and sequencing showed several sequence insertions and deletions along with stretches of novel sequence relative to the previously cloned II/9A locus. These sequence insertions and deletions are present throughout development, are not tissue-specific, and are all located on the same DNA molecule. We believe they represent a population polymorphism found in *Sciara.* Heterozygous adults and homozygous adults for the polymorphisms have been observed. Any specific DNA sequences or spacing requirements important for regulating amplification of the II/9A locus should be conserved between the two polymorphic sequences. Indeed, a 9 nucleotide sequences present at TP1 and at putative TP2 as well as several putative ecdysone response elements are preserved. Insertions and deletions upstream of the 1 kb origin occur within a conserved sequence. However, some novel sequence stretches are located on the right side of the 1 kb origin region proximal to the gene II/9-1 promoter. This suggests that these sequences may not be as crucial as the upstream sequences.

135

**Does the Ecdysone Receptor Mediate DNA Puff Amplification in the Fly *Sciara*?**

J. Johnson, J. M. Waggener, M. S. Foulk, Y. Yamamoto, M. Ezrokhi, H. Smith, N. Wu, S. A. Gerbi; Mol Bio, Cell Bio, Biochem., Brown Univ. BioMed. Div., Providence, RI

Circumstantial evidence suggests that the ligand-bound transcription factor, ecdysone receptor (EcR), may induce repeated rounds of re-replication, resulting in DNA amplification at DNA puffs of larval salivary gland polytene chromosomes of the fly *Sciara.* DNA sequence analysis of the largest DNA puff at locus II/9A revealed potential binding sites for EcR at the amplification origin (ORI EcRE) as well as at the transcription promoter that is 2.5 kb downstream of the origin. To investigate the possibility of steroid hormone regulation of DNA replication/amplification, we have cloned and sequenced the two isoforms of *Sciara* EcR (EcR-A and EcR-B) and produced polyclonal antibodies against isoform-specific peptides. We also cloned and sequenced *Sciara* ultraspiracle (USP), the heterodimer partner of EcR. Gel shifts showed that the "Ori EcRE" can bind *in vitro* expressed *Sciara* EcR-A/USP and EcR-B/USP heterodimers. We determined the developmental profile of EcR-A and EcR-B expression by Real Time PCR using EcR-A or EcR-B isoform-specific primers; we sequenced DNA encoding *Sciara* 18S rRNA to use as a normalization control. EcR-A and EcR-B mRNAs were relatively constant in whole animals throughout development, with a slight (~ 1.5 fold) increase in expression observed for EcR-A during *Sciara* mid-larval and early pupal stages and an (~ 3 fold) increase of EcR-A expression in pupal stages. The expression levels of EcR-A and EcR-B in *Sciara* salivary glands was similar to that in whole larvae, but there was a more pronounced increase in EcR-A at mid-larval stage (~8 fold) and also at the early pupal stage (~8 fold). The developmental profile of protein expression for *Sciara* EcR-A and EcR-B mirrored that of mRNA expression. The increase in EcR-A in salivary glands at amplification stage implied a possible role for this hormone receptor in DNA re-replication.

136

**Ecdysone Induces *Sciara* Puff II/9A DNA Amplification**

M. Foulk, Y. Yamamoto, C. Liang, D. Alam, S. A. Gerbi; Mol Bio, Cell Bio, Biochem., Brown Univ. BioMed Div., Providence, RI

DNA puffs of *Sciara* salivary gland polytene chromosomes undergo DNA amplification, overriding the control that an origin fires only once per round of genomic replication. We have focused on DNA puff II/9A which amplifies ~17 fold. Our data suggest that DNA amplification is controlled by the steroid hormone, ecdysone. Salivary glands from pre-amplification stage larvae were cultured in Cannon's medium with and without ecdysone. Subsequently, Southern blots were probed for DNA puff II/9A and the unamplified III/9B RNA puff as a control. Determination of the ratio between the signals revealed the fold amplification. Salivary glands amplified about 2-fold after one day of culture and about 12-fold after two days, indicating that ecdysone is involved in amplification. There was no amplification after incubation in medium lacking ecdysone. Cycloheximide or actinomycin D inhibited ecdysone induced amplification. Salivary glands from post-amplification stage larvae could not be induced to amplify further. To study this induction *in vivo*, larvae from pre-amplification stages were injected with 32 ng of ecdysone or 50%

ethanol (mock). Subsequently, the amplification level in genomic salivary gland DNA was assessed by quantitative PCR using primers specific to DNA puff II/9A and RNA puff III/9B. Amplification of the II/9A locus was determined by comparison to the ratio of the signal of II/9A:III/9B in adult DNA (ratio in adult = 1). In contrast to mock-injected larvae (no amplification: 1.2 +/-0.35), the ecdysone-injected larvae demonstrated a range of amplification up to 12.9 +/- 2.75 fold. The level of ecdysone-induced amplification generally correlated with the extent of the pre-pupal phenotype. These results suggest that ecdysone plays a role in the induction of DNA amplification. Further work will determine if this function is direct, as suggested by putative ecdysone response elements at the DNA puff II/9A origin.

137

**Regulation of Cell Cycle Progression in Immortalized Human Mammary Epithelial Cells by Eukaryotic Initiation Factor 4E**

S. Li, O. Issaenko, S. Avdulov, R. McMullen, M. Peterson, P. Bitterman, V. Polunovsky; Pulmonary Medicine, University of Minnesota, Minneapolis, MN

Eukaryotic translation initiation factor 4E (eIF4E) is the mRNA cap binding protein and is rate limiting for translation initiation. Many human malignancies, including human breast cancer, over express eIF4E, which is associated with a poor prognosis. Previously we found that over expression of eIF4E confers chemically immortalized human mammary epithelial cells (HMEC) with two crucial hallmarks of malignant conversion - autonomy for survival and growth signals. We extended these initial experiments using HMECs harboring the hTERT gene to examine mechanisms governing translationally regulated autonomy in cells with a defined genetic constitution. Here we show that over expression of eIF4E in HMEC/hTERT produces cells that form transformed foci on plastic in sparse culture and grow in an anchorage-independent manner in agar. These cells have a reduced requirement for extracellular signals to survive and to progress through the cell cycle when cultivated in chemically defined medium supplemented with insulin and/or EGF. Ectopic expression of eIF4E in growth factor deprived cells increased cell cycle entry in response to insulin and EGF, and accelerated G0-to-S transit of the stimulated cells. Moreover, in contrast to their non-transduced counterparts, a subpopulation of HMEC/hTER/eIF4E cells synthesized DNA even in the absence of growth factors. These alterations are associated with elevated phosphorylation of Rb at cdk4/6 and cdk2 specific sites, as well as with increased levels of cyclins D1 and E. Our observations show that up-regulated eIF4E acts as a proto-oncogene that mimics growth factor signaling by activation of a subset of key G1/S checkpoint regulators. Thus, hyperactivation of the cap-dependent translation apparatus can function as a specific component of the malignant transformation pathway, which promotes clonogenic expansion by reducing cell dependence on growth and survival signals and a proper substratum.

138

**Function of Human Polo Like Kinase-2 in Centriole Duplication**

I. Hoffmann,[1] O. Cizmecioglu,[1] S. Duensing[2]; [1]DKFZ, Heidelberg, Germany, [2]Dept. of Molecular and Cell Biology, Pittsburgh, PA

Centrosomes contain a pair of centrioles that duplicate once during the cell cycle to give rise to two mitotic spindle poles, each containing one old and one new centriole. Centriole duplication initiates at the G1/S transition in mammalian cells, and is completed during S and G2 phase. The localization of a number of protein kinases to the centrosome has revealed the importance of protein phosphorylation in controlling the centrosome duplication cycle. The centrosome cycle needs to be tightly coordinated with the cell division cycle. A central part of our ongoing studies is the functional characterization of the human polo-like kinase (Plk) family member Plk2. Plk2 kinase activity peaks during the G1 phase of the cell cycle. Plk2 localizes to the centrosome and is involved in the regulation of centriole duplication. Two characteristic sequence motifs in Plks, the polo boxes, are required for targeting the respective kinase to various structures during cell cycle progression. We find that kinase dead Plk2 (Plk2-NA) functions as competitor of endogenous Plk2 for centrosomal binding. However, kinase-dead Plk2 with a point mutation in the polo box region (Plk2-NAWF) reveals a reduced binding to the centrosome suggesting that binding occurs in a polo box dependent manner. Furthermore, using a GFP-centrin1 cell line we find that Plk2 localizes to the mother centriole during G1 phase and then localizes to both mother and daughter centriole during S phase. siRNA-mediated down-regulation of Plk2 leads to mitotic spindle abnormalities as an apparant failure of duplication of centrioles. To further assess the function of Plk2 in both centriole duplication and G1-Phase, we have performed a screen for Plk2 substrates. We will report our progress in the characterization of these substrates in centrosome function.

139

**Regulation of G1/S Phase Transition and APCCdh1 by Reactive Oxygen Species**

C. G. Havens, S. F. Dowdy; Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA

Dividing cells have a higher metabolic rate than quiescent cells and metabolism has been suggested to influence cell proliferation. To investigate the role of metabolism in cell cycle progression we examined cell size, mitochondrial mass and reactive oxygen species (ROS) levels in highly synchronized cell populations progressing from early G1 to S phase. We found that ROS increased dramatically compared to cell size and mitochondrial mass. Since ROS have been shown to play a role in cell proliferation and transformation, we hypothesized that ROS could contribute to cell cycle progression. Therefore, using antioxidants, we prevented the increase in ROS and found that cells arrested in late G1 after transition across the restriction point, with active cyclin E:cdk2 kinase, and inactive hyperphosphorylated pRb. However, cells failed to accumulate cyclin A protein and therefore activate cyclin A:cdk2 kinase, even in the presence of cyclin A mRNA. Further examination revealed that cyclin A was ubiquitinated by the Anaphase Promoting Complex (APC) and degraded in a proteasome dependant manner. Furthermore, the antioxidant arrest could be rescued by overexpression of Emi1, an inhibitor of APC. This work demonstrates that the accumulation of a critical level of endogenous ROS are necessary for inactivation of the Anaphase Promoting Complex to allow S phase progression.

140

**Novel Function of Muskelin in Cell Cycle Progression**

M. Valiyaveettil, J. C. Adams; Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

Adhesion of normal cells to extracellular matrix is important for their progression through the cell cycle. This regulation acts mainly in the G1 phase of the cell cycle. Muskelin is an intracellular protein originally characterized by its ability to increase cell spreading on the extracellular matrix protein, thrombospondin-1. Muskelin is a multidomain protein that is a unique and conserved member of the kelch-repeat superfamily of

proteins [Biochem. J. (2004), 381, 547-559]. Muskelin is present throughout the cell-cycle, with increased levels in late G1 and S phase. The objective of this work is to analyze the hypothesized functional role of muskelin in the cell cycle, using over- and under-expression studies. GFP- or FLAG- tagged muskelin were over-expressed in muskelin-negative Cos-7 cells and the cell cycle profile analyzed by flow cytometry after 72h. Compared to GFP-expressing cells, over-expression of muskelin resulted in a significant increase in cells with 4n DNA content. Domain deletions were used to map activity to the kelch-repeats and C-terminus. Microscopic analysis indicated a point of inhibition during entry into mitosis. Stable expression of shRNA vectors was used to deplete endogenous muskelin in A549 human lung epithelial cells. In 4 clonal cell lines analysed with $\geq$ 60% depletion of muskelin, the proportion of cells with 2n DNA content was significantly increased. In these lines, cells were flatter with altered, discontinuous, mobile ruffles. At molecular level, the cells had a normal content of cyclin D1, but had reduced hyperphosphorylated Rb (retinoblastoma protein), with partial reduction in phosphorylation at S780 and severe reduction of phosphorylation at S807/S811. Cyclin A protein was also reduced. These studies implicate muskelin in the regulatory network upstream of Rb phosphorylation that controls cell cycle progression into S phase.

141

### A Septin-dependent Pathway for Bud Morphogenesis during G1

T. A. Egelhofer, D. R. Kellogg; MCD Biology, UCSC, Santa Cruz, CA

The septins are a family of conserved proteins that have been implicated to function late in the cell cycle, where they have been shown to play a role in cytokinesis and controlling cell cycle progression during G2/M. However, many septin alleles show synthetic lethal interactions with the G1 cyclins *CLN1* and *CLN2*, suggesting that the septins may also play an earlier role in the cell cycle. To understand the role septins play during G1, we created a *cln1Δcln2Δ* strain with a temperature sensitive allele of *CDC12*. When these cells were synchronized in early G1 and released at the restrictive temperature, the cells were unable to form buds with a normal morphology and they largely failed to direct growth to the daughter bud. We next tested whether Shs1, a non-essential septin, is also synthetically lethal with *cln1Δ cln2Δ*. We constructed a *shs1Δcln1GAL-CLN2* strain that grew normally on galactose, but failed to grow on dextrose. Most of the cells arrested as either large unbudded cells or with small, poorly formed buds. In addition, many of the cells failed to localize the other septins, while others showed diffuse septin localization over one end of the cell or weak septin staining at the bud neck. These results suggest that Shs1 functions in a unique pathway that plays a role in bud morphogenesis and septin function. Since Shs1 is required for viability only in the absence of Cln1 and Cln2, it may function in a pathway that is initiated by the other G1 cyclins, *PCL1* and *PLC2*. We found that Shs1 phosphorylation was significantly reduced in *pcl1Δpcl2Δ* cells upon release from a G1 arrest. Taken together, these results suggest that the septins play an important role early in the cell cycle to regulate the pattern of growth.

142

### Knockdown of Human Mcm10 Activates G2 Checkpoint Pathway

J. Park,[1] K. Lee,[1] D. Hwang[2]; [1]Institues of Molecular Biology and Genetics, Seoul National University, Seoul, Republic of Korea, [2]Department of Biological Sciences, Seoul National University, Seoul, Republic of Korea

MCM10 is required for DNA replication initiation by recruiting Cdc45 and DNA polymerase alpha in *S. pombe* and *X. laevis*. To investigate the roles in human DNA replication, we performed hMCM10 siRNA experiment. The siRNA treatment increased the cells in G2/M. No increase of phospho-H3 suggests that the treated cells are arrested in G2 phase rather than M phase. This arrest appears to be caused by G2 checkpoint activation through Wee1 pathway. Our results suggest that the certain level of hMCM10 is necessary for completion of chromosome replication and progression of cell cycle to mitosis.

## Actin (143-150)

143

### A Single Pointed End Mutation Abolishes Polymerization in Yeast Actin

D. J. Teal, J. F. Dawson; Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

The development of nonpolymerizing actin mutants is central to obtaining crystal structures depicting actin dynamics. While it was thought that polymerization-defective actins could only be coexpressed in yeast cells, we show that yeast can express a non-polymerizing actin mutant as its sole source of actin. Yeast actin possessing an A204C mutation (AC-actin) at the pointed end does not polymerize when purified, even at 0.1 mM concentration. Yeast cells expressing AC-actin grow slower than wild type cells (3 hours versus 2.5 hours doubling times) with cold and osmolarity sensitivity and possess abnormal F-actin cables. Monitoring light scattering at 360 nm resulted in no observable polymerization with 5 uM AC-actin. Electron micrographs of AC-actin polymerization reactions revealed no filaments, ruling out the formation of short filaments. Polymerization of 5 uM AC-actin is rescued in the presence of 5 uM phalloidin. Partial polymerization of 5 uM AC-actin in the presence of 7.5 uM non-muscle tropomyosin was observed, while no polymerization of 10 uM AC-actin was seen in the presence of 10 uM myosin subfragment-1. Copolymerization experiments containing 5 uM wild type yeast actin and 0, 1.6, 5, or 15 uM AC-actin exhibited a similar maximum concentration of F-actin, suggesting that only wild type actin participated in filament formation. The elongation rate increased with increasing concentrations of AC-actin, suggesting that AC-actin participated in the formation of nuclei from which wild type yeast actin polymerized from the free barbed end. This hypothesis is supported by the slight decrease in maximal F-actin concentration with increased AC-actin concentration, suggesting the presence of increased numbers of shorter filaments. This evidence supports the hypothesis that AC-actin acts as a pointed-end nucleating and capping protein and that AC-actin is a candidate for structural studies including important actin binding proteins.

144

### Hydrophobic Loop Mutant F-actin Stability/Instability due to Disulfide Bond Formation

D. C. Scoville,[1] J. Stamm,[1] D. Toledo Warshaviak,[1] A. Shvetsov,[1] P. Rubenstein,[2] W. Hubbell,[1] E. Reisler[1]; [1]Biochemistry and Molecular Biology, UCLA, Los Angeles, CA, [2]Biochemistry, University of Iowa, Iowa City, IA

It has been postulated that the hydrophobic loop of actin (residues 262-274) swings out and assists in actin filament formation and stabilization. We have shown previously that “docking” of this loop to the monomer surface via intramolecular disulfide bonds in yeast actin mutant

L180C/L269C/C374A in G- or F-actin prevents the formation of filaments or destroys them, respectively (Shvetsov, 2002). Here, we analyzed F-actin dynamics utilizing four hydrophobic loop mutants that have cysteine residues introduced at a single location along the yeast actin loop. Lateral, copper-catalyzed disulfide cross-linking of the mutant cysteine residues to the native C374 in the neighboring strand within the filament was fastest for S265C, followed by V266C, L267C, and L269C, respectively. This data is not consistent with the Holmes model of F-actin, but is reinforced by electron paramagnetic resonance (EPR) measurements, showing that S265C lies closest to C374 within the filament, followed by V266C, L267C, and L269C, respectively. Single cysteine EPR analysis of each of the four mutant residues within the loop demonstrated the mobility of the S265C residue, whereas the V266C, L267C, and L269C residues were progressively less mobile within F-actin. Electron microscopy revealed normal filament morphology for the cross-linked mutant S265C and V266C filaments. However, cross-linking destabilized L267C and L269C filaments; only very short filaments could be observed in electron micrographs. These results illustrate the inherent dynamics of the 262-274 loop, including its ability to populate conformational states that destabilize the filament. Such states may be exploited within a cell by filament destabilizing factors. Research supported by grants from the NSF and NIH.

145

**The Real-Time Monitoring of the Folding State of Tetramethylrhodamine Labeled Actin**

A. A. Perieteanu, B. Sweeting, J. F. Dawson; Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Current methods to measure the folding state of actin generally involve techniques such as circular dichroism, differential scanning calorimetry, or fluorescence requiring cost-intensive instrumentation. Chemical modification of actin with 5'-tetramethylrhodamine (TMR-actin) has been used to visualize filament growth, cytoskeletal interactions, and protein structure. We show that the folding state of actin can be monitored using a change in absorbance properties of TMR-actin employing visible light at 557nm. Using this absorbance as an assay, the relative stability of TMR-labeled skeletal muscle actin was determined in the presence of $Mg^{2+}$, $Ca^{+2}$, and various nucleotides, as well as varying TMR-actin concentrations. The melting temperature ($T_M$) of $Ca^{+2}$-ATP TMR-actin was concentration dependent, however at concentrations above 5 uM a consistent $T_M$ of 62.55 ± 0.1ºC was measured, compared to 62.50 ± 0.6ºC measured for unlabelled actin using an independent assay. Mixing experiments showed that the ratio of TMR-actin to wild type actin in solution did not affect the measured $T_M$. We observed that that $Mg^{+2}$-ATP TMR-actin is less thermostable than $Ca^{+2}$-ATP TMR-actin in both filamentous and globular forms. We monitored the polymerization kinetics and filament stability for various ratios of TMR-actin:actin copolymers. Stabilizing and destabilizing effects of gelsolin segment 1, DNAse I , myosin S1, tropomyosin, and phalloidin were observed for globular actin and filamentous actin. An advantage to using this simple and inexpensive method is its ability to directly analyze the folding state of TMR-actin without interference from actin binding proteins. This method shows increased sensitivity over other spectroscopic methods, and allows for the thermal analysis of TMR-actin at lower concentrations than commonly used methods such as differential scanning calorimetry.

146

**Conformational States of the Nucleotide Cleft in Actin**

D. S. Kudryashov, D. T. Warshaviak, E. Reisler; Chemistry and Biochemistry, UCLA, Los Angeles, CA

Actin is one of the most abundant and conserved eukariotic proteins. The polymerization of actin, which is accompanied by ATP hydrolysis, is a driving force for many cellular processes, including cell migration. Actin recognition by regulatory proteins depends on the state of ATP hydrolysis rendering these states critical to the regulation of actin-based motility. It has been suggested that the nucleotide cleft in actin opens upon ATP hydrolysis and Pi release, but this was questioned by others. Recently we demonstrated that the solution methods used for examining the cleft conformation yield ambiguous results. To probe directly the cleft and monitor changes in the intra-cleft distances, we introduced cysteins at each side of the nucleotide cleft of yeast actin and substituted the reactive Cys374 with serine (CCS mutant). The resulting mutant supports yeast growth at rates similar to wild type actin cells, indicating that the in vivo functional properties of the mutant are not perturbed. Most of the in vitro properties of the CCS mutant are similar to these of wild type actin. This includes the rates of tryptic and subtilisin digestion of the subdomain 2 region, the nucleotide exchange rates under all conditions tested (CaATP, MgATP, and MgADP), and nucleotide exchange inhibition by gelsolin segment 1 and DNase I. These observations suggest strongly that the CCS mutant of yeast actin is a good model for monitoring conformational changes in the cleft region. Using homobifunctional thiol cross-linking reagents, we found that the shorter span reagents cross-link actin with increasing efficiency in the following order of states: Mg-ADP-G-actin, Ca-ATP-G-actin, Mg-ATP-G-actin, and Mg-ATP-phalloidin stabilized F-actin. Although, these data do not yield definitive distances within the cleft, they reveal the tendency of actin to adopt an increasingly closed cleft conformation in the above order of states.

147

**Evidence for Four Steps in the Thermal Denaturation of F-Actin**

C. R. Gicquaud, B. Heppell; Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, PQ, Canada

With the development of differential scanning calorimeter (DSC), the *in vitro* study of the unfolding process of proteins has become a subject of increasing interest. Several of these studies were done with actin, where domains of this molecule have been suggested and interactions with ligands characterized. However, the previous studies done with actin, as with many other proteins, were carried out at only one scanning rate and at one protein concentration. The objective of the present work is to analyze the thermal denaturation of F-actin with DSC under different experimental conditions. The steps revealed by the thermograms were identified by CD spectroscopy and probe fluorescence. We found that changing the protein concentration and the scanning rate completely modified the aspect of the thermograms, casting doubt on previous conclusions. We were able to resolve the thermal denaturation of actin into four steps. The first is centered at 45°C, and the fluorescence of pyrene-actin suggests that it is a depolymerization or a fragmentation; this step is reversible. The second step at 56°C, accompanied by an increase of the fluorescence of ε-ATP, is probably a release of the nucleotide from its binding site; this second step is irreversible. The third step at 57-64°C is a change in the environment of the tryptophanes, as shown by the intrinsic fluorescence. The fourth step is the unfolding of the peptide chain, as revealed by CD spectra and ANS fluorescence. From the thermograms, we have measured the calorimetric enthalpy for the unfolding of F-actin to be 64 megaJ/mole, (1 400 J/g), a value much larger than those obtained for other proteins. Since denaturation and stability are closely related, this particularity of actin could be an adaptation of its role as a protein that transmits forces.

148

**Rapid and Reversible Optical Switching of Protein Fluorescence in Living Cells**

S. Mao,[1] T. Sakata,[1] T. Gomez,[2] G. Marriott[1]; [1]Physiology, University of Wisconsin-Madison, Madison, WI, [2]Anatomy, University of Wisconsin-Madison, Madison, WI

We present a new approach to bring about rapid and reversible, optical switching of protein fluorescence within living cells. We have developed a family of thiol reactive optical switches that undergo rapid and reversible transitions between a colorless spiro (SP) state and a colorful merocyanine (MC) state - the two states of the switch are efficiently controlled through alternate excitation of SP with 365 nm light (SP to MC), and MC with 543 nm light (MC to SP) - unexpectedly, MC is shown to decay to the ground state with the emission of red (620-650 nm) fluorescence. The advantages of our optical switch conjugates for switching protein fluorescence over other approaches include: 1), Optical transitions between the SP and MC states are rapid and fully reversible and can be realized using 1-photon and 2-photon (SP to MC) excitation; 2), Optical transitions between SP and MC do not release photoproducts; 3), The absorption of the MC to SP transition can be tuned over a broad wavelength range (>500~650 nm). We show that cells loaded a BIPS-actin conjugate exhibit MC fluorescence (650nm) when excited with 543 nm light. Stronger illumination of the MC-conjugate with 543 nm light generates the non-fluorescent SP-actin; on the other hand, excitation of SP-actin with a short pulse of 365 nm light regenerates the fluorescent MC-actin. The cycle of optical switching between SP- and MC-actin can be repeated over many excitation cycles (>20) with high fidelity and shows little evidence of fatigue. The ability to use light to rapidly and reversibly modulate MC-fluorescence of a protein conjugate should prove useful in FRAP/photoactivation of fluorescence and speckle microscopic analyses of dynamic cellular processes.

149

**Orientation of F-actin in the Contractile Ring in Fission Yeast**

T. Kamasaki,[1,2,3] M. Osumi,[2,4] I. Mabuchi[3]; [1]Department of Oral Histology, School of Dentistry, Showa University, Tokyo, Japan, [2]Division of Material and Biological Function Sciences, Graduate School of Science, Japan Women's University, Tokyo, Japan, [3]Division of Biology, Graduate School of Arts and Sciences, University of Tokyo, Tokyo, Japan, [4]Institute of Medical Mycology, Teikyo University, Tokyo, Japan

Cytokinesis in animal and primitive eukaryotic cells is executed by constriction of the contractile ring which is formed underneath the plasma membrane at the division plane. F-actin and myosin-II are main components of the contractile ring, but little is known about the arrangement of F-actin in the ring. Here, we show the entire arrangement of F-actin decorated with myosin subfragment 1 in the contractile ring in fission yeast cells. In equatorial serial sections of the whole ring, numbers of actin filaments oriented reversely from each other were roughly 1:1. These filaments were not homogeneously mixed in the ring, but those showing the same directionality tended to cluster in the ring. The average length of each filament was less than 1 μm indicating that the actin filaments are staggered in the ring. This study provides us with an insight into how the contractile ring is formed and how it contracts.

150

**Modulation of the Actin Cytoskeleton of the Anterior Pituitary Folliculostellate Cells TtT/GF by Pro-Inflammatory Cytokines**

S. Solinet, M. L. Vitale; Pathology and Cell Biology, Université de Montréal, Montréal, PQ, Canada

In the anterior pituitary, the folliculostellate (FS) cells are agranular cells with distinct functions such as support for endocrine cells, intercellular communication, phagocytosis and modulation of the endocrine and immune responses. We have shown that the FS cell shape responds to the hormonal milieu. Because FS cells are the target of cytokines, here we studied the effect of pro-inflammatory cytokines on their morphology. We used a stable mouse cell line, the TtT/GF cells, which show morphological and biochemical features of normal FS cells. TtT/GF cells were treated with the pro-inflammatory cytokines TNFα (20 ng/ml), IL-1β (10ng/ml) and IFNγ (10U/ml) for increasing periods of time (0 to 96hs). The shape of the cells, the reorganization of the actin cytoskeleton and changes in the localization and expression of actin-binding proteins were evaluated by fluorescence microscopy and Western blot. Treatment with TNFα or IL1-β but not INF-γ induced apoptosis of TtT/GF cells. The cells rounded up, detached and the nuclei showed condensed chromatin. Twenty % of TtT/GF cells evidenced signs of apoptosis after 24hs-treatment with TNFα or IL-1β. By 96hs the percentage increased to 50%. Caspase 8 was activated by 48hs of treatment with TNFα or IL-1β while caspase 3 was activated by 72hs. In immunofluorescence, incubation with TNFα or IL-1β for 72hs caused the loss of proteins involved in focal contacts such as α-actinin, vinculin and paxillin. In addition, cytokines caused the cleavage of the actin-anchoring protein, spectrin. The results show that 1) the pro-inflammatory cytokines TNFα and IL-1β but not IFN-γ caused changes in the FS cell morphology that were related to the induction of apoptosis, and 2) actin-anchoring proteins were down regulated and/or cleaved during this process. Supported by NSERC-Canada.

## Actin-Associated Proteins I (151-171)

151

**Actin Capping Protein Alpha Maintains *Vestigial*-expressing Cells in the *Drosophila* Wing Disc Epithelium**

F. Janody,[1] J. Treisman[2]; [1]LGPD, Developmental Biology Institute of Marseille, Marseille, France, [2]Department of Cell Biology, Skirball Institute, NYU School of Medicine, New York, NY

*Drosophila* appendages develop from larval imaginal discs, which are composed of a pseudostratified columnar epithelium apposed to the thin squamous peripodial membrane. Patterning and growth of these structures are established by a series of complex regulatory interactions among transcription factors and morphogens. In a mosaic genetic screen, we identified mutations in the *capping protein alpha (cpa)* and *twinstar (tsr)* genes, which encode Actin Binding Proteins (ABP). Cpa binds to the fast growing ends of actin filaments, blocking further addition of subunits. *tsr* encodes the Cofilin homologue, which severs actin filaments and promotes depolymerization. Interestingly, clones mutant for *cpa* cannot be recovered in the epithelium of the wing pouch territory. Mutant cells undergo an epithelial to mesenchymal transition, extrude basally and die by apoptosis. However, *cpa* mutant cells persist in the epithelium of the remainder of the wing disc. In contrast, *tsr* mutant clones proliferate and survive equally well in all regions of the wing disc. Both *cpa* and *tsr* restrict actin filament polymerization throughout the cell cortex, indicating that excessive actin polymerization is not sufficient to explain the cpa mutant phenotype; rather, *cpa* is specifically required to maintain epithelium integrity in the wing pouch territory. The expression of *vestigial (vg), which* promotes growth of the wing pouch epithelium, is not affected in *cpa*

mutant clones. Misexpression of vg in *cpa* mutant cells is sufficient to cause them to extrude basally and die in all regions of the wing disc. This shows that *vg* controls the requirement for *cpa* and suggests that regulation of the actin cytoskeleton by ABP has distinct effects on different epithelial territories.

152

**Binding of V-1 to Actin Capping Protein**

S. Ghosh, D. Sept; Biomedical Engineering, Washington University, St. Louis, MO

V-1 (also known as myotrophin) is a 12 kDa protein consisting of three ankyrin repeats. Proteomic studies have revealed that V-1 interacts directly with capping protein (CP). Interestingly, unlike other CP regulators such as $PIP_2$ and CARMIL, V-1 cannot uncap already capped barbed ends but can only inhibit capping. Deletion mutants have shown that the C-terminus of the ß-subunit of CP plays an important role in the binding of V-1 to CP. However the molecular details of the interaction of V-1 with CP are unknown. Through the use of molecular dynamics and molecular docking simulations, we have developed a model of how V-1 binds CP. Our results indicate that V-1 binds both to the C-terminus tentacle of the ß-subunit of CP and the body of CP reducing the mobility of the tentacle and thus reducing the affinity of CP for the barbed end of actin filaments. These results agree well with the observation that the beta tentacle is critical for CP function.

153

**Binding of PI(4,5)$P_2$ to Capping Protein**

M. E. McCully, D. Sept; Biomedical Engineering and Center for Computational Biology, Washington University, St. Louis, MO

Phosphatidylinositol-4,5-biphosphate (PIP2), a lipid present in the cell membrane, has been found to regulate actin filament polymerization by binding to actin-regulating proteins. In the presence of PIP2, capping protein can be removed and inhibited from further capping, allowing the free barbed end to grow against the cell membrane. Experimental data has suggested a binding location for PIP2 in the C-terminal region of the α-subunit, but the molecular details of the interaction remain unknown. Through a combination of molecular dynamics and molecular docking simulations, a binding location was identified at the base of the α-C-terminus. Our results suggest specific interactions between the negatively charged phosphate groups of PIP2 and the three positively charged residues: Lys 256 and Arg 260 from the α-subunit and Arg 255 from the β-subunit. Experiments introducing point mutations at these residues have confirmed this binding location.

154

**Characterization of a Low Abundant Profilin Isoform in *Dictyostelium discoideum***

R. Arasada,[1] A. Gloss,[1] J. Faix,[1] A. A. Noegel,[2] M. Schleicher[1]; [1]A.-Butenandt Institute for Cell Biology, Ludwig-Maximilian University Munich, Munich, Germany, [2]Center for Biochemistry, Medical Faculty, University of Cologne, Cologne, Germany

Profilins are small and ubiquitous G-actin binding proteins that are necessary for the turnover of actin filaments. Profilin forms a 1:1 complex with ADP-actin, exchanges ADP with ATP thus regenerating the pool of ATP-actin needed for polymerization. Apart from actin, it also binds to phosphatidylinositols and many other proteins with stretches of proline residues. These include also actin binding proteins such as the Arp2/3 complex, formins, and VASP Like most of the eukaryotic organisms tested, *Dictyostelium discoideum*, a facultative single celled social amoeba, contains two profilin isoforms (Haugwitz *et al*., 1994, Cell 79:303-14). The completed genome sequence of *D. discoideum* (Eichinger *et al*., 2005, Nature 435:43-57) revealed a third profilin isoform. Recombinant profilin III behaves nearly identical to the other two profilin isoforms *in vitro*, and rescues the phenotype of pI/II minus cells when overexpressed. Interestingly, profilin III is uniformly expressed throughout development, and about 200-fold less abundant than profilin I and II. This was also the reason that the protein could not be detected in pI/II-minus cells. Immunolocalization studies using profilin III specific polyclonal antibodies in vegetative cells show an overall distribution and sometimes a strong enrichment at the tips of filopodia. In contrast to profilin I or II, the third profilin isoform interacts with VASP in a yeast two hybrid assay. To further characterize the function of profilin III, we generated profilin III null mutants. Null mutants show reduced efficacy in migration towards a cAMP source during chemotaxis whereas development is not affected. The data suggest that this low abundant profilin isoform *in vivo* is not primarily an actin sequestering protein but affects cell migration during chemotaxis as a regulator in signal transduction.

155

**Competitive Biochemical Interactions of Myotrophin/V-1 with Actin Capping Protein and NF-kappa B**

N. Bhattacharya, J. A. Cooper; Cell Biology, Washington University School of Medicine, St. Louis, MO

In cells, nuclear factor kappa B (NF-κB) signaling can accompany and depend on actin assembly. Actin capping protein (CP) regulates actin assembly and cell motility. The protein V-1 / myotrophin interacts with CP and with NF-κB. In the present study, we demonstrate that V-1 binds directly to CP, with a Kd of ~ 100 nM. V-1 binding inhibited the ability of CP to cap the barbed ends of actin filaments, but did not cause uncapping. The C-terminal tentacle of the CP beta subunit, which is known to bind to actin, was necessary and sufficient to bind V-1. Two loops of V-1, which extend out from the α-helical backbone of this ankyrin repeat protein, were necessary for V-1 to bind CP. Parallel computational studies determined a bound conformation of the beta tentacle with V-1 that is consistent with these findings. We found that V-1 binds directly to NF-κB, with differential affinity in the submicromolar range for the p50 homodimer, p65 homodimer and p50 / p65 heterodimer forms. NF-κB and CP compete with each other for binding to V-1. These interactions provide a biochemical pathway connecting actin assembly with NF-κB signaling, which may have physiological relevance.

156

**Mammals Have Two Twinfilin Isoforms; Twinfilin-1 and Twinfilin-2**

E. M. Nevalainen,[1] M. Vartiainen,[2] T. Matilainen,[1] M. Salminen,[1] A. Braun,[3] R. Fässler,[3] P. Lappalainen[1]; [1]Institute of Biotechnology, Helsinki, Finland, [2]Cancer Research UK, London Research Institute, London, United Kingdom, [3]Max Planck Institute for Biochemistry, Martinsried, Germany

Twinfilin is a highly conserved actin monomer-binding protein that regulates cytoskeletal dynamics in organisms from yeast to mammals. Twinfilin binds ADP-G-actin with a higher affinity than ATP-G-actin and efficiently inhibits actin filament assembly *in vitro*. In addition to actin monomers, twinfilin also binds capping protein and this interaction may localize twinfilin -actin monomer complexes to the sites of rapid actin

filament assembly in cells. Unlike yeasts, worms and flies, which have only one twinfilin protein, we identified two twinfilin isoforms, twinfilin-1 and twinfilin-2, from mammals. The two mouse twinfilin isoforms bind actin monomers and capping protein with similar affinities to each other, but show cell-type specific expression patterns. Twinfilin-1 is the major isoform in developing embryos and in adult mouse non-muscle cells, whereas twinfilin-2 is the predominant isoform in differentiated muscle cells. Furthermore, our cell biological studies suggest that the subcellular localizations of twinfilin-1 and twinfilin-2 are regulated through distinct signaling pathways. To reveal the biological roles of the two mammalian twinfilins, we have generated knock-out mice for both twinfilin isoforms through targeted disruption of the twinfilin-1 and twinfilin-2 genes. These knock-outs will help to resolve the role of twinfilin in actin dynamics in mammalian cells as well as their specific roles in various actin-dependent cellular and developmental processes in mammals.

157

**Molecular Architecture of Twinfilin and its Actin-Monomer Binding Regions**

V. O. Paavilainen, M. Hellman, M. Koskinen, P. Permi, A. Annila, P. Lappalainen; Institute of Biotechnology/University of Helsinki, Helsinki, Finland

Actin machinery is regulated by a variety of actin regulating proteins. Of these only six actin-monomer binding proteins are conserved in eukaryotes. They include the profilin, ADF/cofilin, twinfilin, SRV2/CAP, WASP/WAVE and verprolin/WIP -proteins. Twinfilin is a novel and ubiquitous actin-monomer binding protein composed of two ADF-H (Actin Depolymerizing Factor -Homology) domains, separated by a short linker and followed by a 35-residue C-terminal tail region. Twinfilin binds the actin monomer with a 1:1 stoichiometry, inhibits the nucleotide exchange on actin and also inhibits monomer assembly into filaments. Twinfilin appears to first associate with the actin monomer via its' N-terminal 'low-affinity' ADF-H domain, after which it is transferred to the 'high-affinity' C-terminal ADF-H domain. The depletion of twinfilin in S.cerevisiae results in defects in the bipolar budding pattern and in rough eye phenotype and aberrant bristle morphology in Drosophila melanogaster. These phenotypes arise because of uncontrolled actin polymerization in the absence of twinfilin. To properly understand the molecular mechanisms of twinfilins' function, it was imperative to gain structural information of the twinfilin molecule. For this purpose we had earlier solved the crystal structure of the N-terminal ADF-H domain, and have now solved the C-terminal domain structure by NMR methods. The new structure is highly similar to the N-terminal ADF-H domain and other known ADF-H domains. We are currently confirming the actin-monomer binding site of twinfilins' C-terminal ADF-H domain by systematic mutagenesis. The two structures combined with biochemical data will enable us to better understand twinfilins' role as an essential regulator of actin dynamics.

158

**Twinfilin Severs Actin Filaments and Promotes Actin Turnover In Vivo**

J. B. Moseley,[1] K. Okada,[1] D. R. Kovar,[2] H. I. Balcer,[1] T. D. Pollard,[2] B. L. Goode[1]; [1]Biology, Brandeis University, Waltham, MA, [2]Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT

Working in concert multiple actin binding proteins regulate the dynamic turnover of actin networks. Until now, only one well-conserved factor has been identified that directly promotes actin filament disassembly, ADF/cofilin. Here, we demonstrate a related cellular function for the highly conserved actin binding protein twinfilin, which until now was thought to function primarily as a monomer sequestering protein. Twinfilin localizes *in vivo* to sites of rapid actin turnover, including cortical patches in yeast and membrane ruffles in mammalian cells. We show that purified budding yeast twinfilin (Twf1) actively promotes filament disassembly in bulk kinetic assays and dramatically reduces average filament length in seconds. Further, we use real-time evanescent wave fluorescence microscopy to show that Twf1 severs individual actin filaments to generate free filament barbed ends. Consistent with these biochemical functions, we demonstrate that Twf1 promotes actin filament turnover in live yeast cells. Previous work has demonstrated that capping protein (Cap1/2) binds directly to Twf1 *in vitro* and recruits Twf1 to sites of rapid actin turnover *in vivo.* However, no specific function has yet been ascribed to this physical interaction. We demonstrate that Cap1/2 inhibits Twf1 severing activity. Further, filament severing and depolymerization by Twf1 are tightly regulated *in vitro* by pH. Thus, twinfilin defines a novel class of filament severing and depolymerization protein that is subject to regulation by both pH and capping protein. These results suggest that twinfilin coordinates multiple key aspects of actin dynamics--filament severing, filament capping, and monomer sequestering--at sites of rapid actin turnover *in vivo*.

159

***Caenorhabditis elegans* Kettin, a Large Immunoglobulin-like Repeat Protein, Binds to Filamentous Actin and Is Involved in Actin Organization in Muscle**

S. Ono, K. Mohri, K. Ono; Pathology, Emory University, Atlanta, GA

Kettin is a large actin-binding protein with immunoglobulin-like (Ig) repeats, which is associated with the thin filaments in arthropod muscles. Here, we report identification of kettin in the nematode *Caenorhabditis elegans* and its functional characterization. We found that MH44, one of the monoclonal antibodies that were raised against *C. elegans* muscle proteins [G. R. Francis and R. H. Waterston. (1985) *J. Cell Biol.* 101: 1532-1549], specifically reacts with kettin (Ce-kettin). We determined the entire cDNA sequence of Ce-kettin that encodes a protein of 472 kDa with 31 Ig repeats. Arthropod kettins are splice variants of much larger connectin/titin-related proteins. However, the gene for Ce-kettin (*ketn-1*) is independent of other connectin/titin-related genes. Ce-kettin localizes to the thin filaments near the dense bodies in both striated and non-striated muscles. The C-terminal four Ig repeats were sufficient for binding to actin filaments *in vitro*, whereas the adjacent non-Ig region augmented the actin-binding activity. RNA interference of Ce-kettin caused partial disorganization of the actin filaments in body wall muscle. These results indicate that kettin is an important regulator of actin organization and suggest that kettin might be functionally homologous to vertebrate cytoskeletal proteins with Ig repeats, such as palladin, myopalladin, and myotilin.

160

**Calcium/Calmodulin Dependent Protein Kinase-II Interacts with Flightless-I in Embryonic Fibroblasts**

M. E. Seward,[1] J. H. Moxley,[1] M. W. Mayhew,[2] A. F. Horwitz,[2] R. M. Tombes[1]; [1]Department of Biology, Virginia Commonwealth University, Richmond, VA, [2]Department of Cell Biology, University of Virginia, Charlottesville, VA

CaMK-II is a highly conserved and broadly expressed $Ca^{2+}$/calmodulin-dependent protein kinase. CaMKII is believed to be involved in many cellular processes including synaptic plasticity, gene expression, cell cycle progression, cell adhesion, and motility. In an effort to identify binding partners potentially regulated by CaMK-II in motile fibroblasts, mass spectrometry was performed on immunopurified FLAG-tagged isozymes of CaMK-II (α, β, $\delta_c$, and $\delta_e$). Identified binding partners included not only CaMK-II and calmodulin, but also myosin subunits, cofilin, tropomodulin-3, β-tubulin, β- and γ-actin, flightless-I (Fli-I), and LRRFIP (a Fli-I binding protein). Of particular interest is Fli-I, which is a gelsolin-related actin-binding protein Fli-I and was identified in association with both full length CaMK-II and with CaMK-II lacking the catalytic domain. Fli-I is a developmentally essential 145kDa protein that is conserved throughout all metazoans. It is known to shuttle between the nucleus and the cytosol, where it is involved in regulating actin filaments. The interaction of endogenous CaMK-II with Fli-I was shown through reciprocal immunoprecipitations in differentiating mouse neurons as well as in mouse and human embryonic fibroblasts. For instance Fli-I IPs contained CaMK-II, as determined through both activity assays and immunoblots. Similarly, CaMK-II IPs contained the expected 145kDa band when blotted for Fli-I. These findings indicate that Fli-I associates with CaMK-II through either its variable or association domain and raises the possibility of CaMK-II dependent regulation of Fli-I.

161

**Alternative Exon Choice in the Gamma Tropomyosin (γ-Tm) Gene is Not Absolutely Programmed in Different Cell Types**

J. Hook,[1,2] G. Schevzov,[1,2] B. Vrhovski,[1] F. Lemckert,[1] T. Borovina,[1] E. Hardeman,[3] P. Gunning[1,2]; [1]Oncology Research Unit, The Children's Hospital at Westmead, Westmead NSW, Australia, [2]Discipline of Paediatrics and Child Health, University of Sydney, Sydney NSW, Australia, [3]Muscle Development Unit, Children's Medical Research Institute, Westmead NSW, Australia

The actin filament system is fundamental to cellular functions including regulation of shape, motility, cytokinesis, intracellular trafficking and tissue organisation. Tropomyosins (Tm) are highly conserved actin binding proteins differentially affecting stability and function of actin filaments. The mammalian Tm family consists of four genes; α-Tm, β-Tm, γ-Tm and δ-Tm. Multiple Tm isoforms are generated by alternative splicing, and expression of these isoforms is highly regulated during development. γ-Tm nonmuscle gene products (Tm5 NM1-11) are involved with the establishment of neuronal polarity, axon outgrowth and maturation in brain. Alternative exon choice in γ-Tm products can lead to intracellular targetting to diverse locations such as growth cones versus axons in neurons and Golgi versus stress fibres in fibroblasts. Using the γ-Tm gene, we tested mechanisms that underpin alternative exon choice in development. We first removed the amino-terminal exon 1b to ablate all isoforms. Genetic analysis failed to identify homozygous knockout mice, indicating γ-Tm gene products are essential for embryonic development. We next restricted γ-Tm gene product repertoire and forced alternative exon selection by removing carboxy-terminal exons 9c and 9d. Mice lacking exon 9c show a normal phenotype with elevated expression of products from exon 9a in specific tissues. Mice lacking exon 9d display compensating use of exons 9a and 9c, however no compensation for loss of 9d is observed in embryonic stem cells. We conclude that selection of alternative exons is not absolutely fixed and that compensatory use of alternative exons can differ between cell types.

162

**Genetic and Biochemical Analyses Predict a Mechanism for Aip1p in Actin Filament Disassembly**

M. G. Clark, B. Haarer, D. C. Amberg; Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, NY

Regulation of the organization and function of the actin cytoskeleton requires rapid filament turnover and modulation of turnover rates by accessory factors. Biochemical analyses indicate that cofilin and Aip1p cooperate to accelerate actin dynamics. These conserved proteins co-localize in cortical actin networks from diverse organisms. Cofilin has long been known to promote depolymerization of actin filaments by accelerating pointed-end disassembly and by exhibiting a weak filament-severing activity. Aip1p dramatically enhances disassembly of actin filaments in a cofilin-dependent manner by capping the barbed ends, activating severing, or a combination of both. PCR-induced mutagenesis has allowed us to identify active sites on Aip1p required for the actin and cofilin interactions, leading to a model for the ternary complex formed by Aip1p, cofilin, and actin. Biochemical and genetic analyses of these mutants have been conducted in an effort to elucidate the mechanism of Aip1p's activity by identifying mutations that disrupt the ability of Aip1p to participate in severing- and/or capping-specific filament disassembly. In addition, we have combined an *aip1Δ* allele with a sub-array of gene deletion alleles, whose gene products are known to localize to cortical actin patches in yeast. By identifying synthetic growth defects that occur when *aip1Δ* is combined with other gene deletions alleles, we were able to make inferences regarding specific physiological roles for Aip1p in the cell. Our findings suggest that Aip1p participates in a specific step of the endocytic internalization process.

163

**AFAP-110: A Lyn Kinase Activator**

I. H. Qazi, D. C. Flynn; Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV

In many invasive or metastatic cancers, activated c-Src kinase is found at elevated levels and causes a transformed phenotype where there is a disruption of the actin cytoskeleton, reduced growth requirements, etc. In many cases increased activity is due to enhanced activation of c-Src by interacting proteins rather than mutations or an increase in transcription or translation. AFAP-110 is an adapter protein that was first identified as a SH3/SH2 binding partner for activated c-Src. A dominant positive form of AFAP-110 (AFAP-$110^{\Delta L.zip}$) helped demonstrate its intrinsic ability to activate c-Src via domain interactions and lead to subsequent cellular transformation. Due to high sequence and structural homology between Src family members it is hypothesized that the AFAP-110 could similarly activate Lyn kinase. While expression of Lyn is restricted primarily to hematopoietic tissues, recent work on tumor samples such as glioblastoma multiforme and hormone refractory prostate cancers implicate Lyn as a culpable player in their carcinogenesis. Here we demonstrate that the SH3 domain of Lyn was sufficient to affinity absorb GFP-tagged AFAP-110 from fibroblast cell lysates and the two proteins interact when co-transfected into epithelial and fibroblast cells. Both stable and transient transfections of Lyn and AFAP-$110^{\Delta L.zip}$ into SYF cells seems to activate Lyn in a similar pattern to that of c-Src. These patterns included changes in the actin cytoskeleton, increased cellular tyrosine phosphorylation, the redistribution of cellular cortactin, and the formation of podosomes structures. These findings may have clinical relevance in tumors where AFAP-110 may receive upstream signals to activate Lyn kinase.

164

**Caldesmon is an Integral Component of Podosomes in Smooth Muscle Cells**

B. A. Webb, R. Eves, A. S. Mak; Biochemistry and Protein Function Discovery Program, Queen's University, Kingston, ON, Canada

Podosomes are highly dynamic, actin-based structures commonly found in motile and invasive cells such as macrophages, osteoclasts, and vascular smooth muscle cells. Here we investigated the role of caldesmon, the actin-binding protein, in the formation of podosome in smooth muscle A7r5 cells induced by phorbol-ester, PDBu. We found that endogenous l-caldesmon, which was normally localized to actin-stress fibers and membrane ruffles, was recruited to PDBu-induced podosomes. Over-expression of l-caldesmon caused dissociation of actin-stress fibres and disruption of focal adhesion complexes, but did not lead to formation of podosomes. Treatment of the caldesmon-transfected cells with PDBu induced the formation of caldesmon-enriched podosomes. Transiently transfected N-terminal fragment of l-caldesmon, Cad40, containing the myosin-binding sites, was not present in PDBu-induced podosomes; whereas the C-terminal fragment, Cad39 housing the binding sites for actin, tropomyosin and calmodulin, was translocated to podosomes. The caldesmon mutant, CadCamAB, which does not interact with $Ca^{2+}$-calmodulin, was not recruited to PDBu-induced podosomes. These results suggest that l-caldesmon is required for PDBu-induced podosome formation and is an integral part of the core actin column. The calmodulin-binding domain at the C-terminal half of l-caldesmon is essential for the translocation of l-caldesmon to the podosomes.

165

**Pyrin, Product of the MEFV Locus, Interacts with Actin Polymerizing Machinery**

A. L. Waite,[1] P. Schaner,[2] N. Richards,[1] B. Balci,[1] D. L. Gumucio[1,2]; [1]Cell & Developmental Biology, University of Michigan, Ann Arbor, MI, [2]Cell & Molecular Biology, University of Michigan, Ann Arbor, MI

Familial Mediterranean Fever (FMF) is an autoinflammatory disorder characterized by attacks of fever with pain and neutrophil infiltration localized to the abdomen, chest, skin, or joints. FMF is caused by mutations in the *MEFV* locus, the protein product of which is pyrin. Pyrin is expressed primarily in neutrophils and monocytes. Exon 1 of pyrin encodes a pyrin domain (PyD), which in other proteins is associated with inflammation and apoptosis. In monocytes, pyrin is primarily cytoplasmic and localizes to leading edge lamellipodia, a site of active actin polymerization. We confirmed that pyrin is localized to polymerizing actin using the *Listeria* rocket assay. Mutant pyrin also localizes to rockets. In a yeast two-hybrid screen, we identified PSTPIP1, a known actin-associated protein, as a pyrin-binding protein. Though we hypothesized that PSTPIP1 could be responsible for recruiting pyrin to *Listeria* rockets, we found that PSTPIP1 does not associate with rockets. Thus, another factor must be responsible for the recruitment of pyrin to sites of actin polymerization. We therefore tested whether pyrin binds to any of eight proteins known to be involved in actin polymerization (actin, Arp3, VASP, α-actinin, profilin, cofilin, gelsolin, and capping protein). We found that wild-type pyrin binds to both cofilin and gelsolin. Recently it was discovered that these two proteins are involved in modulating the release of DNase I from actin (Chhabra, 2005), and therefore may mediate the change in a cell from a state of migration to one of apoptosis. Mutant pyrin does not bind to gelsolin but binds to capping protein. This may indicate a change in the essential function of the protein and lead to a condition where inflammatory cells migrate more efficiently but cannot die.

166

**Identification of a Human Mps1 Interacting Protein**

C. P. Mattison, M. Winey; MCD Biology, CU-Boulder, Boulder, CO

Defects in centrosome duplication lead to chromosome instability, aneuploidy, and are found in many types of tumor cells. The mono-polar spindle 1 (Mps1) protein kinase is highly conserved among eukaryotes, and recent studies have shown that human Mps1 (hMps1) is involved in the duplication of centrosomes. To better understand the role of hMps1 in centrosome duplication I performed a two-hybrid experiment with the amino-terminus of hMps1. From this screen I have identified the hMps1 interacting protein 1 (MIP1) gene. The MIP1 gene is conserved among vertebrates and encodes a protein with a large central coiled coil domain and a calponin homology domain at the carboxy terminus. Results with a MIP1 clone fused to Green Fluorescent Protein (GFP) indicate that MIP1 protein localizes to actin filaments in several human cell types. In addition, MIP1 protein binds hMps1 in vitro, and is an in vitro substrate for hMps1. I plan to determine whether MIP1 plays a role in centrosome duplication, and how the interaction with hMps1 contributes to this role. hMps1 has also been shown to be important for the spindle assembly checkpoint, cytokinesis and cell cycle progression. Based upon the sequence and localization of MIP1 to actin filaments, it may be involved in some aspect of cytokinesis. Future experiments will focus on defining the interaction between hMps1 and the AMIP protein in human cells.

167

**The Actin Binding Property of SWAP-70, a Mediator of Membrane Ruffling after Growth Factor Stimulaion**

S. Ihara, Y. Fukui; Applied Biological Chemistry, University of Tokyo, Tokyo, Japan

SWAP-70 is a PH (pleckstrin homology) domain containing protein, which exhibits binding ability to $PIP_3$, a product of PI3-kinase. It has been shown that SWAP-70 is required for efficient membrane ruffling after growth factor stimulation. We previously described that the extreme C-terminal region of SWAP-70 binds to F-actin and that a mutant SWAP-70 lacking the region behaves as a dominant negative reagent. These results suggest that the direct binding of SWAP-70 to F-actin is required for the membrane ruffling. In this study, the property of actin binding activity was further explored. When non-muscle actin was used in co-sedimentation assay, C-terminal region of SWAP-70 exhibited a binding activity much stronger than that to muscle F-actin with an approximately 1:1 stoichiometry in saturation. Therefore, the binding of SWAP-70 to F-actin is isospecific. A mutant SWAP-70 lacking the C-terminal region still exhibited a binding activity, but it was not isospecific. We found that the PH domain sedimented with either type of F-actin at the same extent, suggesting that the PH domain is an additional F-actin binding domain. Although the significance of this binding activity remains unclear, it is interesting to speculate that the two F-actin binding domains play different roles in F-actin organization.

168

**The Nck-Interacting Kinase NIK Phosphorylates ERM Proteins: A Mechanism for Lamellipodia Formation by Growth Factors**

M. Baumgartner,[1] E. M. Blackburn,[2] A. Sillman,[1] J. Srivastava,[1] J. W. Schilling,[2] J. H. Wright,[2] D. L. Barber[1]; [1]Cell and Tissue Biology,

University of California, San Francisco, San Francisco, CA, [2]Sugen, South San Francisco, CA
The mammalian Ste20-like Nck-interacting kinase NIK and its orthologs Misshapen in *Drosophila* and Mig-15 in *Caenorhabditis elegans* have a conserved function in regulating cell morphology. NIK substrates that control cell morphology, however, have not been clearly identified. We now report that the ERM proteins ezrin, radixin, and moesin are substrates for NIK, and that NIK phosphorylation of ERM proteins is necessary for growth factor-induced lamellipodia formation. ERM proteins regulate cell morphology and plasma membrane dynamics by anchoring actin filaments to integral plasma membrane proteins. ERM proteins are activated by phosphorylation of a conserved C-terminal threonine that induces a conformational change promoting their binding to F-actin. *In vitro* assays show that purified NIK phosphorylates the conserved C-terminal threonine in ERM proteins (T567 in ezrin). Additionally, NIK directly binds the N-termini of ERM proteins and the ERM-binding site is within the proximal 33 amino acids of the NIK C-terminal regulatory domain. In fibroblasts, expression of a truncated NIK containing only the N-terminal kinase domain and the ERM-binding domain was sufficient to increase phosphorylation of ERM proteins. NIK and ERM proteins co-localize at the distal margin of lamellipodia, and in fibroblasts and epithelial cells increased phosphorylation of ERM proteins in lamellipodia by platelet-derived growth factor and epidermal growth factor is inhibited by expression of kinase-inactive NIK-D152N. In contrast, NIK-D152N has no effect on increased phosphorylation of ERM proteins by thrombin. Live cell imaging shows that lamellipodia extension in response to growth factors is inhibited by expression of NIK-D152N or ezrin-T567, but not by expression of wild-type NIK or ezrin. Hence, direct phosphorylation of ERM proteins by NIK constitutes a signaling mechanism controlling growth factor-induced membrane protrusion and cell morphology. Supported by NIH grant GM47413.

169

**Cell Swelling Causes Actin Reorganization and Recruitment of the Actin Binding Protein Moesin in Membrane Protrusions in Collecting Duct Principal Cells**

G. Tamma, M. Svelto, G. Valenti; Department of General and Environmental Physiology, University of Bari, Bari, Italy
Cell swelling causes actin cytoskeleton remodelling associated with the modification of cell architecture. The ERM family (ezrin, radixin, moesin) proteins crosslink the actin filaments and plasma membrane and are important signal transducers during actin reorganization. The actin cytoskeleton is a dynamic structure regulated by the monomeric G protein of the Rho family. Moesin plays a key role in actin remodelling by binding actin filaments through its C-terminal domain and RhoGDI through its N-terminal domain. In collecting duct CD8 cells hypotonicity-induced cell swelling resulted in a deep actin reorganization, consisting in loss of stress fibers and the formation of F-actin patches in membrane protrusions. Immunofluorescence studies revealed that actin and moesin co-localized in membrane protrusions. TritonX-100 extraction procedure, which preserves the cytoskeleton and the cytoskeleton-associated proteins, confirmed that cell swelling increased the interaction between actin and moesin. Hypotonic stress caused recruitment of moesin in cell cortex and a transition of moesin from the oligomeric to monomeric functional conformation. In its monomeric form, moesin can bind interacting proteins when the C and N terminal domain are exposed and this conformation is stabilized by phosphorylation of a conserved threonine in the C-terminal domain by PKC or Rho kinase. Interestingly, exposure of CD8 cells to hypotonic stress increased the amount of threonine-phosphorylated moesin. While PKC inhibitor, Ro-31-8220, did not affect moesin phosphorylation, Rho kinase inhibitor Y27632 decrease the basal level of the phosphorylated moesin but did not affect the hypotonicity-induced increase of phosphorylated moesin. Altogether the data represent the first evidence that hypotonicity-induced actin remodelling is associated with moesin recruitment at the cell border and interaction with actin in a phosphorylated state.

170

**EGFP-CALI of Mena Changes Lamellipodial Dynamics**

E. Vitriol,[1] J. E. Bear,[1,2] K. Jacobson[1,2]; [1]Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC
Chromophore assisted light inactivation (CALI) is a tool for local and instantaneous loss of protein function. CALI first requires the attachment of a chromophore to a target protein; inactivation is then mediated by the photogeneration of reactive oxygen species (ROS). The short half-life of ROS largely limits inactivation to the targeted protein. Enhanced green fluorescent protein can be used for CALI and has the advantage of being both genetically encoded and covalently linked to the protein target of interest. Here we tested EGFP-CALI in a well-defined live cell system: the effect of Mena/VASP proteins on lamellipodial dynamics. When Mena/VASP members are present at the leading edge protrusions emerge rapidly and are transient; by contrast, when Mena/VASP members are absent from the leading edge the protrusions emerge at a slower velocity and become more persistent (Bear et al. *Cell*. 109(4):509-21), resulting in faster migration rates (Bear et al. *Cell*. 101(7):717-28). To perform EGFP-CALI of Mena, we used Mena/VASP -/- fibroblasts ($MV^{D7}$ cells) rescued with EGFP-Mena; using rescued knockout cells allowed us to avoid a potential loss of the CALI phenotype by the presence of endogenous, unlabeled protein. Employing kymograph analysis we show that local irradiation of EGFP-Mena at the leading edge significantly reduces protrusion velocity and increases protrusion persistence. This effect is reversible, with lamellipodial dynamics returning to their original state following fluorescence recovery into the irradiated region. Irradiation of non-rescued $MV^{D7}$ cells or $MV^{D7}$ cells expressing EGFP alone did not change leading edge dynamics. This work was supported by the NIH Cell Migration Consortium (GM 64346).

171

**Investigation of Expression and Biological Roles of the F-Actin Associated Protein Coronin 3**

C. S. Clemen, A. Rosentreter, A. Hasse, Z. Spoerl, M. Stumpf, A. A. Noegel; Institute of Biochemstry I, Medical Faculty, University of Cologne, Cologne, Germany
Coronin proteins belong to the fundamental and versatile WD40-repeat domain proteins. All coronin proteins possess a conserved basic N-terminal motif and three to ten WD40-domain repeats clustered in one or two core domains. Generally, they are mainly found at the submembraneous area, bind F-actin in vitro, and are implicated in eukaryotic F-actin dynamics in vivo or they have a role in vesicular transport. Coronin 3 is a widely expressed 57 kDa protein being abundant in the central nervous system. Its N-, WD40-, and partially the C-domain are highly conserved in mammals and within different members of the protein family. The very C-terminal coiled-coil domain mediates the formation of trimers, the entire C-terminus binds to and cross-links F-actin and confers membrane association in vitro and in vivo. Cytosolic coronin 3 shows phosphorylation

which most likely regulates the subcellular localization and biological activity. Transfection studies employing various coronin 3 domain constructs revealed that for its localization and biological function an interaction of N- and C-termini are required as well as regulatory effects mediated by the central WD40-domain. The protein is involved in processes like neurite outgrowth, noradrenaline secretion, wound healing, cytokinesis, proliferation, and cell motility. During early murine brain development and the first postnatal stages all areas of the brain express coronin 3. Postnatally, the expression in the grey matter decreases along with neuronal differentiation, except for hippocampal pyramidal and dentate gyrus neurons, and cerebellar Purkinje cells, while levels in the white matter increase in the course of myelination. Our results favor a major role for coronin 3 in cellular processes depending on F-actin dynamics.

## Unconventional Myosins (172-197)

172

### The Globular Tail Domain Regulates the Enzymatic Activity of Myosin V

K. Thirumurugan,[1] P. J. Knight,[1] J. A. Hammer,[2] J. R. Sellers[3]; [1]School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom, [2]Laboratory of Cell Biology, National Heart, Lung and Blood Institute, Bethesda, MD, [3]Laboratory of Molecular Physiology, National Heart, Lung and Blood Institute, Bethesda, MD

Myosin V is a processive motor that translocates various cargo along actin filaments. It has two heads with neck domains containing 6 IQ-motifs that bind calmodulin. The actin-activated MgATPase of tissue purified myosin V is strongly regulated by calcium. In the absence of calcium the ATPase activity is low and the molecule exists in a compact, folded state that sediments at 14S in the ultracentrifuge. Addition of micromolar levels of calcium activates the ATPase activity 10-30 times and is associated with unfolding of the molecule (11S). EM observation of negatively stained myosin V in the absence of calcium shows a compact, isosceles-triangular structure (1). Using single particle image processing techniques we find that the long sides of the triangular structure correspond to the elongated neck domains of myosin V and that the shorter base of the triangle is formed by the motor domains interacting with the globular tail domain (GTD). A segment of the coiled-coil tail is seen running from the junction of the two necks to the GTD. This structure is consistent with our observation that baculovirally-expressed myosin V HMM which retains the two-headed structure, but lacks the GTD is constitutively active regardless of calcium concentration. Moreover, we find that a GST-GTD fusion protein inhibits the actin-activated MgATPase of myosin V HMM to about 25% of its maximal activity with a Ki of about 1 uM. This is consistent with the model proposed above where, in the absence of calcium and cargo, myosin V folds into a compact structure where the globular tail domains directly bind the motor domains to stabilize an inhibited structure. 1. Wang et al. J.Biol.Chem. 279: 2333-2336, 2004.

173

### Dynamics and Distribution of Myo5c, a Class V Myosin of Epithelial Cells

D. T. Jacobs, R. E. Cheney; Cell and Molecular Physiology, University of North Carolina-Chapel Hill, Chapel Hill, NC

Class V myosins are hypothesized to function as actin-based organelle transporters. The third member of the class V myosin in mammals, myosin 5c, is expressed chiefly in epithelial cells (Rodriguez and Cheney, JCS, 115:991-1004). Our results show Myo5c is abundant in exocrine tissues such as the lacrimal gland, pancreas, and mammary gland. Immunohistochemistry reveals that Myo5c localizes to the apical domain of the polarized epithelial cells in these tissues. We are using MCF-7 and HeLa cells as model systems to identify the organelles and trafficking pathways associated with Myo5c. Immunocytochemistry shows that Myo5c antibodies label discreet puncta as well as tubules that span tens of μm through the cytoplasm in MCF-7 cells. The puncta are often closely associated with the plasma membrane near the leading edge of migrating MCF-7 cells. When transfected into MCF-7 cells, full-length GFP-Myo5c exhibits a punctate and tubular distribution similar to endogenous Myo5c. Wide-field epi-fluorescence imaging of live cells reveals that the tubules associated with GFP-Myo5c undergo long-range, highly dynamic movements at ~ 0.5 μm/sec. Nocodazole treatment (5μM) causes a dramatic loss of these cytopasmic tubules, demonstrating a dependence upon microtubules. Live-cell imaging using TIRF microscopy reveals that a sub-population of GFP-Myo5c puncta are closely associated with the ventral plasma membrane and undergo slower movements of ~0.03 μm/sec. The movements of these puncta are insensitive to nocodazole treatment, suggesting a dependence on f-actin. Our results suggest that Myo5c is associated with one or more organelles that are transported by microtubules and also interacts with f-actin at the cell cortex. (Supported by APS Porter Fellowship to DTJ and NIH/NIDCD grant DC03299 to REC)

174

### A Putative Role for Mammalian Class V Myosins in Transcription

A. J. Lindsay, M. W. McCaffrey; Biochemistry, University College Cork, Cork, Ireland

The two class V myosins in yeast, Myo2p and Myo4p, have been shown to play functional roles in a number of cellular processes, including. These include the regulation of proper mitochondrial, vacuolar, and ER inheritance during cell division, orientation of the mitotic spindle, mRNA transport and secretory vesicle transport. To date the three mammalian class V myosin isoforms, MyoVa, MyoVb, and MyoVc, have solely been implicated in membrane transport. We became interested in MyoVb when it was reported to interact with the small GTPase Rab11, and its interacting partner Rab11-FIP2. We generated a MyoVb-specific anti-peptide antibody, and were surprised to observe that in addition to labelling cytoplasmic vesicles it also displayed strong nuclear localisation, by immunofluorescence. We also observed a similar pattern for MyoVa using a commercial antibody. The nuclear localisation of both MyoV isoforms was confirmed biochemically. The observation that MyoVb was present in nucleoli where it colocalises with RNA polymerase I, and newly synthesised rRNA, led us to believe that it plays a role in transcription. Indeed, its nuclear localisation pattern was altered by transcription inhibitors. In contrast, MyoVa is excluded from the nucleolus and is unaffected by these inhibitors. As well as labelling vesicles, MyoVa also localised to P-bodies, cytoplasmic structures that are involved in mRNA degradation. We therefore believe that in addition to membrane transport these unconventional myosins may also be involved in regulating transcription and RNA turnover.

175

### Intracellular Dynamics of *Drosophila* Myosin-V

J. Toth, J. R. Sellers; Laboratory of Molecular Physiology, NHLBI, Bethesda, MD

We have studied the cellular role of myosin-V, an actin-based cytoplasmic motor, in *Drosophila*. The vertebrate and yeast myosin-Vs have been shown to associate with the endoplasmic reticulum, vacuoles and protein-RNA complexes through cargo specific adaptor molecules to function as organelle transporters. Vertebrate myosin-Va is a processive motor capable of carrying cargo for μm distances as a single molecule. Conversely, we have recently reported that the fly myosin-V is not processive therefore it has to function in ensembles. Such difference in the motor mechanism may reflect a different role in cytoplasmic transport. We created stable *Drosophila* S2 cell lines expressing full-length myosin-V or its C-terminal cargo binding domain fused to GFP. We performed confocal live cell imaging to investigate the intracellular dynamics of the overexpressed proteins. We used immunocytochemistry to determine the endogenous cellular distribution of myosin V in S2 cells. Our experiments show that in S2 cells, the endogenous myosin-V is mainly associated with the nuclear envelope and partially co-localizes with the endoplasmic reticulum but not the Golgi compartments. We observed partial co-localization with both filamentous actin and microtubules. In live cells, the full-length overexpressed protein exhibits diffuse cytoplasmic distribution dotted with vesicle-like moving structures. The cargo-binding tail fragments, on the other hand, concentrate mainly in bright dots that move unidirectionally in the cytoplasm at a speed of 0.32 ± 0.18 μm/s. These structures continue moving after the addition of actin or microtubule depolymerizing agents. Our results suggest that the fly myosin-V is associated with yet unidentified, potentially ER-derived vesicles that are transported on both the actin and microtubule networks. In this respect, *Drosophila* myosin-V functions similarly to other described vesicle transporter myosins.

176

**3-D Structure of the Inhibited State of Myosin V from Cryoelectron Tomography of 2-D Arrays**

J. Liu,[1] D. W. Taylor,[1] E. B. Krementsova,[2] K. M. Trybus,[2] K. A. Taylor[1]; [1]Institute of Molecular Biophysics, Florida State University, Tallahassee, FL, [2]Department of Molecular Physiology and Biophysics, University of Vermont, Burlington, VT

Myosin V (myoV) is a 2-headed molecular motor responsible for cargo transport along actin filaments in brain. In addition to its active state, myoV has a compact conformation formed in low [$Ca^{2+}$] that has low actin activated ATPase activity. We have formed 2-D arrays on lipid monolayers of recombinant full length myoV from mouse brain and used cryoelectron tomography of ice embedded arrays to obtain the first 3-D image of the inhibited conformation. The arrays have a hexagonal unit cell of dimension 65.3 nm but long range order is poor and the arrays have numerous defects. To obtain a resolution of 2.4 nm where an atomic model could be constructed, we used correspondence analysis to identify similar molecular structures within the arrays for subsequent averaging. The 3-D image has sufficient resolution to resolve the two lobes of the calmodulin light chains and to visualize the initial segment of the coiled-coil domain. The SH3 domain is clearly visible in the reconstruction facilitating unambiguous positioning of the myosin V motor domain crystal structure in the atomic model. The 3-D image reveals a compact conformation with the coiled-coil domain folded back between the two heads. Extra density that we assign to the cargo-binding domain is positioned on the motor domains in the vicinity of the ATP binding pocket. The actin binding interfaces are unobstructed by the cargo binding domain and the lever arm is oriented in a postpower stroke position typical of strong actin binding states. ATPase inhibition appears to be due to inhibition of nucleotide exchange. The structure suggests that motor recycling after cargo delivery occurs, not by diffusion, but through transport on actively treadmilling actin filaments. Supported by NIAMSD.

177

**Myosin Vc and Cytoplasmic Dynein are Enriched in Membrane Compartments in Lacrimal Acinar Epithelial Cells**

R. Marchelletta,[1] D. Jacobs,[2] R. Cheney,[2] J. A. Garner,[1] S. Hamm-Alvarez[1]; [1]University of Southern California, Los Angeles, CA, [2]University of North Carolina, Chapel Hill, NC

Acinar epithelial cells of the lacrimal gland are responsible for production and regulated release of tear proteins. A subset of membrane vesicles are enriched in myosin Vc (MyoVc). To help elucidate the function of MyoVc in this population, we have examined the vesicles for other constituents that may be co-localized. Primary rabbit lacrimal acini were isolated and cultured for 3 days. Confocal fluorescence microscopy and Nycodenz density gradient analysis were used to evaluate distributions of MyoVc, dynein, $p150^{Glued}$, the polymeric immunoglobulin A receptor (pIgR) and actin filaments in acini without or with carbachol (CCH, 100 μM) stimulation. For some experiments, acini were transduced on day 2 with replication-deficient adenovirus (Ad) encoding the dominant-negative MyoVc tail, fused to GFP (DN MyoVc) or GFP. The effect of DN MyoVc versus GFP on exocytosis was measured. Confocal fluorescence microscopy revealed MyoVc in association with large (1-2 μM) vesicles beneath the apical membrane in resting and CCH-stimulated acini. Dynein and $p150^{Glued}$ showed extensive colocalization with MyoVc, particularly in CCH-stimulated acini, while pIgR showed partial colocalization. These observations were validated by preliminary subcellular fractionations over Nycodenz density gradients. Functional analysis of the effects of DN MyoVc on secretion in acini transduced to ~90% efficiency revealed a modest (15%) but significant ($p \leq 0.05$, n=10) inhibition of CCH-stimulated β-hexosaminidase and bulk protein release; however, DN MyoVc significantly (($p < 0.05$, n=4) inhibited CCH-stimulated release of SC by ~50%. Confocal microscopy and biochemical studies support the enrichment of MyoVc and cytoplasmic dynein on the same membranes in lacrimal acini and suggest that MyoVc may facilitate CCH-stimulated SC release.

178

**Characterization of the *S cerevisiae* mRNA Localization Motor Myo4p**

B. D. Dunn, P. A. Takizawa; Cell Biology, Yale University, New Haven, CT

Cell polarity is established by targeting proteins to specific cellular locations. One mechanism to localize proteins is through the intracellular transport and localization of specific mRNAs. Subsequent translation leads to protein localization and cellular asymmetry. Both actin and microtubule based motors have been shown to transport mRNAs, but it is not known how these motors bind to specific mRNAs or how they efficiently move them. In the budding yeast *Saccharomyces cerevisiae*, the class V myosin Myo4p transports ASH1 mRNA to the bud tip. Two additional proteins, She3p and She2p, are required for Myo4p to associate with ASH1 mRNA. To understand how Myo4p transports ASH1 mRNA, we have developed a method to purify active Myo4p from yeast cell extracts. We find that She3p is tightly associated with Myo4p and co-purifies with the motor in an approximate ratio of 1:1. She3p acts as an adaptor linking Myo4p to She2p and the mRNA. We find that the amino-terminal half of She3p binds the predicted coiled-coil and tail domains of Myo4p, while the C-terminal half of She3p interacts with cargo. The *in vitro* rate of Myo4p movement as measured by actin gliding assays is approximately 1 μM/sec, which is consistent with *in vivo* mRNA transport. We have found that Myo4p is a monomeric motor, in contrast to all other known class V myosins. However, Myo4p still maintains a high affinity

for actin in the presence of ATP and has a high duty ratio in actin gliding assays, consistent with other class V myosins. These properties allow Myo4p to transport ASH1 mRNA transcripts to the bud tip rapidly and efficiently.

179

**Myosins Vc and Vb Sequentially Regulate Trafficking of Caveolar and Clathrin-Dependent Endocytotic Membranes**

Y. Chen,[1] L. B. Benseñor,[1] J. A. Mercer,[2] D. W. Provance,[2] J. R. Goldenring,[3] R. E. Cheney,[4] D. Jacobs,[4] G. S. Bloom[1]; [1]Department of Biology, University of Virginia, Charlottesville, VA, [2]McLaughlin Research Institute, Great Falls, MT, [3]Department of Surgery, Vanderbilt University, Nashville, TN, [4]Department of Cell and Molecular Physiology, University of North Carolina, Chapel Hill, NC

Trafficking of caveolar membranes, pigmented organelles and ER involves the cooperative action of actin filaments and microtubules, and myosin Va is an actin motor for pigmented organelle and ER transport. To determine if caveolar trafficking also requires a myosin V, caveolin-1 transport was studied in CHO cells expressing fluorescent dominant negative tail domains of myosin Va, Vb or Vc. All tail domains accumulated in multilamellar cytoplasmic structures, as judged by thin section EM. Caveolin-1 co-localized exclusively with myosin Vc tail, which also co-localized with transferrin receptor (TfR) and the early/sorting endosome marker, Rab5, and inhibited transferrin uptake. In contrast, myosin Vb tail co-localized with TfR, but with neither caveolin-1 nor Rab5. Markers for several other classes of membrane compartments, including late endosomes, lysosomes, autophagosomes and exosomes, failed to accumulate in the myosin Vc-positive compartment, Time lapse imaging of fluorescent versions of caveolin-1, endocytosed transferrin and Rab5 revealed caveolin-1 in both early/sorting and recycling endosomes. These data imply that caveolin-1, converges with transferrin and TfR in early/sorting and recycling endosomes, that progression from early/sorting to recycling endosomes requires myosin Vc, and that caveolin-1 segregates from transferrin and TfR in recycling endosomes before the latter two proteins return to the cell surface by a myosin Vb-dependent mechanism.

180

**The Effects of KE-PDZ Domain on Myosin 18A Distribution and ATPase Activity**

Y. Yang,[1] K. Sader,[2] Q. E. Xu,[1] J. Trinick,[2] J. R. Sellers[1]; [1]Laboratory of Molecular Physiology, National Heart, Lung and Blood Institute, Bethesda, MD, [2]School of Biomedical Sciences, Leeds University, Leeds, United Kingdom

There are at least two alternatively-spliced isoforms of myosin 18A. The α-isoform contains a tandem KE (lys-glu rich) and PDZ domain at its N-terminus (termed KE-PDZ), whereas the β-isoform lacks these motifs. Immuno-histochemical staining and western-blotting suggests that myosin 18A is predominantly expressed in adult mouse brain. Whole mount embryo sections staining reveals myo18A to be predominantly in epithelia cells of various tissues. The KE-PDZ domain is the main localization determinant of myo18A in epithelia cells. Transiently expressed KEPDZ domain distributes along actin filaments in filopodia and lamellipodia. The a-isoform is found in association with filaments, but the β-isoform has a more diffuse localization. Immunoprecipitation of myo18A from mouse brain extracts revealed that it is associated with LC17. We expressed the two truncated isoforms (HMMα and HMMβ) along with LC17in Sf9 cells. Both proteins were purified using the FLAG affinity epitope. EM results show that myo18A is a “two-headed” motor with very short neck consistent with its single IQ motif. HMMα binds to actin in an ATP-independent manner, probably due to the KE-PDZ domain. HMMβ binds to actin in an ATP-dependent manner. Interestingly, both HMMs show weak rigor binding to actin in the absence of ATP. ATPase assays reveal that both isoforms have actin activated-Mg ATPase activity, however the Vmax of α-isoform (0.5 $s^{-1}$) is higher than that of β-isoform (0.07 $s^{-1}$). The $K_{ATPase}$ of α-isoform is 10.1 μM which is 2.4 times lower than that of β-isoform. All the data suggest that KE-PDZ domain involves not only localization of myo18A in epithelia cell, but the regulation of ATPase activity compared to other myosins.

181

**Targeted Disruption of *Myo18B* Results in Myocardial Defects and Embryonic Lethality**

R. Ajima,[1] H. Akazawa,[2] M. Kodama,[3] F. Takeshita,[3] A. Otsuka,[1] M. Tani,[1] T. Kohno,[1] I. Komuro,[2] T. Ochiya,[3] J. Yokota[1]; [1]Biology Division, National Cancer Center Institute, Tokyo, Japan, [2]Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan, [3]Section for Studies on Metastasis, National Cancer Center Institute, Tokyo, Japan

*MYO18B* is a novel unconventional *MYOSIN* gene that was identified from a homozygously deleted region on chromosome 22q in a human lung cancer cell line. Frequent inactivation of this gene in human cancer cells by genetic and/or epigenetic alterations, and suppression of anchorage-independent growth by restoration of its expression suggest that the *MYO18B* gene functions as a tumor suppressor in human carcinogenesis. However, functions of MYO18B in normal cells are still unclear. To investigate the functions of Myo18B *in vivo,* we had generated *Myo18B* gene deficient mice by knocking-in the *nls-LacZ* gene. Deficiency of *Myo18B* in mice resulted in lethal cardiac defects between embryonic day 10.5 (E10.5) and E11.5. LacZ staining of *Myo18B* gene targeted mice tissues or embryos revealed that Myo18B was expressed predominantly in muscles, both in embryonic and adult tissues. We performed immunohistochemical analysis using antibodies against Myo18B to examine the localization of Myo18B in myocytes and muscles. Myo18B localized on F-actin in myocytes and on Z-lines in skeletal and cardiac muscles. Myo18B did not co-localize with type II myosin in skeletal muscles. The localization suggests that Myo18B plays a distinct role from conventional myosins. Furthermore, we performed a reporter assay of *Myo18B* gene promoter to investigate the regulation of *Myo18B* gene expression in myocytes. As expected, the Myo18B promoter was activated by differentiation of C2C12 myocytes. The *Myo18B* gene promoter region contains a myocyte-specific enhancer factor-2 (MEF2) binding site. Mutation of MEF2 binding site decreased basal expression and activation of Myo18B promoter. Thus, it was suggested that MEF2 might regulate Myo18B expression in myocytes. We conclude that Myo18B is a protein that is expressed mainly in muscles and its expression is crucial for cardiac development.

182

**Biochemical and Motile Properties of Myo1b Splice Isoforms**

T. Lin, N. Tang, E. M. Ostap; Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA

Myo1b is a widely expressed myosin-I isoform that concentrates on endosomal and ruffling membranes and is thought to play roles in membrane trafficking and dynamics. Myo1b is alternatively spliced within the regulatory (lever-arm) domain, yielding isoforms with six (myo1b$^{a}$), five (myo1b$^{b}$) or four (myo1b$^{c}$) non-identical IQ motifs. The first three IQs motifs (IQ1-3) are the same for each splice isoform, and splicing occurs

within IQ4-6. Myo1b[a] contains IQ4, IQ5, and IQ6, myo1b[b] contains IQ6 and is spliced within IQ4 and IQ5 resulting in a IQ4/5 hybrid, and myo1b[c] is spliced within IQ4 and IQ6, resulting in the creation of a IQ4/6 hybrid and the loss of IQ5. The calmodulin binding properties of the myo1b IQ motifs have not been investigated, and the mechanical and biological consequences of splicing are not known. Therefore, we expressed the alternatively spliced myo1b isoforms, and we expressed a construct containing a single IQ domain. The constructs were truncated after the final IQ domain and contained a sequence at their COOH-termini that is a substrate for biotin ligase. Site-specific biotinylation allows us to specifically attach the myosin to motility surfaces via a biotin-streptavidin linkage. We measured the ATPase and motile properties of the recombinant myo1b splice isoforms, and correlated these properties with calmodulin binding. We confirmed that calcium-dependent changes in the ATPase activity are due to calcium binding to the calmodulin closest to the motor. We found that calmodulin binds tightly to IQs 1-3 (Kd < 0.2 μM) and very weakly to the alternatively spliced IQ motifs (Kd > 5 μM). Additionally, we found that the motility rate is not linearly related to the number of IQ motifs, suggesting that the calmodulin-saturated regulatory domains of the splice isoforms do not act as rigid lever arms.

183

### Myo1c Binds Tightly and Specifically to $PIP_2$ and $IP_3$

D. E. Hokanson, E. M. Ostap; Physiology, University of Pennsylvania Med School, Philadelphia, PA

Myosin-I is a single-headed, low-molecular-weight member of the myosin superfamily that is thought to associate with acidic phospholipids via its tail domain. Although membrane association is essential for proper myosin-I localization and function, little is known about the physiological relevance of the direct association of myosin-I with phospholipids or about phospholipid head-group binding specificity. To better understand the mechanism of myosin-I-membrane association, we measured effective dissociation constants for the binding of a recombinant myosin-I (myo1c) IQtail construct (which includes three IQ motifs and bound calmodulins) to sucrose-loaded, large unilamellar vesicles (LUVs) composed of phosphatidylcholine (PC) and various concentrations of phosphatidylserine (PS) or phosphoinositol 4,5-bisphosphate ($PIP_2$). We found that the myo1c-IQtail binds tightly to LUVs containing ≥ 60% PS, but very weakly to LUVs containing physiological PS concentrations. Remarkably, the myo1c-IQtail binds tightly to LUVs containing just 2% $PIP_2$, which have a charge equivalent to 6% PS. Additionally, we found that myo1c-IQtail binds to soluble inositol-1,4,5-trisphosphate ($IP_3$) with approximately the same affinity as to $PIP_2$ in LUVs, suggesting that myo1c binds specifically to the head-group of $PIP_2$. An expression construct consisting of the myo1c motor and IQ domains does not bind LUVs containing 2% $PIP_2$, confirming that the $PIP_2$ binding site is in the tail. Finally, we show that a GFP-myosin-I-tail chimera expressed in epithelial cells is transiently localized to regions known to be enriched in $PIP_2$, and that this localization is disrupted by elevated concentrations of intracellular calcium. Our results suggest that myo1c does not bind to physiological concentrations of PS, but rather binds tightly to $PIP_2$.

184

### NMR Structural Study of the *Acanthamoeba* Myosin IC Tail

K. Hwang,[1] F. Mahmoodian,[2] J. M. Gruschus,[1] J. A. Ferretti,[1] E. D. Korn[2]; [1]Laboratory of Biophysical Chemistry, NHLBI, NIH, Bethesda, MD, [2]Laboratory of Cell Biology, NHLBI, NIH, Bethesda, MD

The tail of *Acanthamoeba* myosin IC (AMIC) comprises an N-terminal 220-residue basic region (BR) followed by a 56-residue Gly/Pro/Ala-rich region (GPA1), a 55-residue SH3 domain and a C-terminal 133-residue GPA2 region. Previous cryo-electron microscopy [Ishikawa et al. (2004) PNAS 101, 12189-12194] indicated that the tail was folded back on itself between BR and GPA1 and between GPA1 and GPA2, implying intramolecular interactions among the tail regions. We have initiated NMR studies to determine the structure of the tail and identify specific interactions within it. We expressed three proteins labeled with $^{15}N$ and $^{15}N/^{13}C$: full-length tail, the BR region and the GPA1-SH3-GPA2 segment. $^{15}N/^{13}C$-labeled BR was also expressed in medium containing 65% $D_2O$ to provide higher quality spectra. NMR conditions were optimized for solubility and stability (25 $^{o}$C, 40 mM KCl, 20 mM phosphate, pH 6.5, and 0.5 mM TCEP as a reducing agent). For protein backbone resonance assignment, we used 2D HSQC, $^{15}N$ NOESY, $^{13}C$ NOESY, HNCACB, CBCACONH, and HNCOCAB pulse experiments. The N-terminal basic region showed some β-sheet secondary structure; sequence alignment showed no similar structure in the data base. Superposition of the NMR spectra of the full-length tail with the spectra of the individually expressed BR and GPA1-SH3-GPA2 segments showed some differences, implying that there is some interaction between the N-terminal and C-terminal regions of the full-length tail. We will identify and characterize the flexible, folded and partially folded regions of the full-length tail using the spectra from all three expressed proteins. Furthermore, we will use a homology modeling approach to predict some possible conformationally dependent interactions between the N- and C-terminal halves. We expect these studies will provide additional information on any possible interactions between different regions of the tail of *Acanthamoeba* myosin IC.

185

### Identification and Characterization of Novel Light Chains Associated with the *Dictyostelium discoideum* Class-I Myosins

S. W. Crawley, Y. Yang, L. M. Dryden, A. Bigg, S. P. Smith, G. P. Cote; Biochemistry, Queen's University, Kingston, ON, Canada

The *Dictyostelium discoideum* class I myosins are single-headed, non-filament forming motor proteins composed of a heavy chain and one or more light chains. We have identified and characterized two novel light chains associated with MyoB and MyoD. The light chain associated with MyoD, termed MlcD, is 16 kDa in size and has a typical, calmodulin-like domain structure with four putative EF-hand motifs. In contrast, MyoB contains a small 8-kDa light chain, termed MlcB, that contains only two EF-hand motifs. MlcB binds $Ca^{2+}$ with a $K_d$ value of 0.2 $\mu$M, indicating that it may act as a physiological sensor of *in vivo* $Ca^{2+}$ levels. Mutational analysis shows that the first EF-hand of MlcB is solely responsible for binding $Ca^{2+}$. The solution structure of MlcB in the $Ca^{2+}$-free state is now being solved. MlcB binds to a peptide corresponding to the MyoB IQ motif in the absence and presence of $Ca^{2+}$ with $K_d$ values of 1.2 and 0.5 $\mu$M, respectively. Interestingly, calmodulin binds to the MyoB IQ motif peptide with a similar affinity; however, the binding is strictly $Ca^{2+}$ dependent. Competition assays indicate that MlcB and calmodulin binding to the MyoB IQ motif peptide are mutually exclusive events. The possibility that MyoB can switch light chains in a $Ca^{2+}$-dependent manner is being investigated. Searches of dictyBase (http://www.dictybase.org) show that *Dictyostelium* contain a second small two-EF-hand protein (DDB0219456) that is 37% identical to MlcB. This protein, tentatively termed MlcX, does not bind to the MyoB IQ motif peptide. A FLAG-tagged MlcX has been expressed in *Dictyostelium* and the identification of interacting proteins is currently underway.

186

**Unconventional Myosin 1e Promotes Actin Assembly during Compensatory Endocytosis in *Xenopus* Eggs**

H. E. Yu,[1] W. Bement[2]; [1]Program in Cellular and Molecular Biology, University of Wisconsin -- Madison, Madison, WI, [2]Dept of Zoology, University of Wisconsin -- Madison, Madison, WI

In *Xenopus* eggs, exocytosing cortical granules (CGs) are surrounded by actin "coats", which drive compensatory endocytosis (Sokac *et al.*, Nat Cell Biol. 5: 727-32, 2003). In combination with inositol triphosphate ($IP_3$) uncaging to elevate $Ca^{2+}$ and trigger CG exocytosis, we used time-lapse microscopy to study the role of "unconventional" myosin 1e in the actin coat assembly. Myosins, which bind to filamentous actin, have long been identified as important molecular motors that transport cargos and produce contraction power. Besides the highly conserved motor domain that is present in all myosins, myosin 1e has a long tail that is comprised of 3 myosin tail homology (MyTH) domains. Inhibition of myosin 1e abolished actin coat assembly, suggesting that this myosin actually promotes actin assembly. In addition, our results showed that the MyTH1 and MyTH2 domains of the myosin 1e tail are sufficient for proper myosin 1e recruitment to the exocytosing CGs. More importantly, the MyTH2 domain of myosin 1e preferentially associated with more dynamic actin, giving rise to the possibility that this myosin can target specifically to rapidly assembling actin. Thus, myosin 1e may not only promote actin coat assembly, but may also function to compress the actin coat by tethering the newly assembled actin.

187

**Myo3A Binds Specific Phospholipids via IQ Motif-rich Regions in its Neck and Tail**

A. C. Dosé, A. C. Corsa, B. Burnside; MCB, UC Berkeley, Berkeley, CA

Myo3A is expressed in photoreceptors and ear hair cells. In photoreceptors Myo3A localizes specifically to actin filament bundles of calycal processes. To investigate whether Myo3A binds membrane lipids, we used a protein-lipid overlay assay (PIP strips™, Echelon Inc.). Various Myo3A neck and tail fusion protein constructs were tested. The neck (IQ motifs 1-4) and tail (IQ 5-9) bound to phosphatidylinositol-monophosphates [PI(3)P, PI(4)P and PI(5)P] and phosphatidylinositol-diphosphates [$PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(4,5)P_2$], as well as $PI(3,4,5)P_3$ and phosphatidic acid (PA). To quantify relative affinities for lipids, fusion proteins were tested on PIP arrays, which consist of serial dilutions of phosphoinositol phosphates. Affinities for monophosphates were strongest followed by affinities for di- and triphosphates. A similar pattern was observed for a 23 amino acid fusion protein between IQ motifs 4 and 5. IQ motif 4 alone did not bind phospholipids, nor did the tail domain beyond the last IQ motif. Calmodulin binds to IQ motifs and can compete for lipid binding (as seen for myosin IC). To test whether Myo3A IQ motifs could be affected by the presence of CaM, the neck (1-4) and tail (5-9) IQ domain fusion proteins were overlayed on PIP strips in the presence of 25 υM CaM either with 25 υM $Ca^{2+}$ or 100 υM EDTA. The neck did not bind to $PI(3,4,5)P_3$ or PA in the presence of $Ca^{2+}$ and CaM while the tail binding pattern is unaffected. These data indicate Myo3A binds lipids, mediated at least in part by a region between IQ motifs 4 and 5, and calmodulin. These findings suggest Myo3A may play a role in transporting or localizing lipids within the inner segment and calycal processes of photoreceptors.<br

188

**Myosin VI: An Actin Stabilizer, Not a Cargo Transporter**

T. Noguchi,[1] M. Lenartowska,[2] K. G. Miller[1]; [1]Biology, Washington University in St. Louis, St. Louis, MO, [2]Institute of General and Molecular Biology, Nicolaus Copernicus University, Torun, Poland

To understand the in vivo function of myosin VI, we analyzed the *Drosophila* myosin VI mutant during the individualization stage of spermatogenesis. Actin based machinery consisting of 64 actin cones divides the 64 syncytial spermatids into individual sperm as cones move though whole spermatids. In vitro culture of myosin VI mutant spermatogenic cysts demonstrates that cone movement initiates normally but stops prematurely. The actin cones cannot maintain the normal accumulation of actin during movement. Over expression of myosin VI leads to increase of extra F-actin in the actin cone. S1 labeling demonstrates that actin cone has front half consists of a densely packed meshwork of F-actin and rear half with long actin bundles. In addition, unlike Listeria actin comet tail or lamellipodial actin network, the vast majority of F-actin have their pointed ends facing forward of the cone. In myosin VI mutants, the F-actin density is much less, and the meshwork in the front half of the cone is much smaller. Myosin VI localizes to the front edge of the cone where a number of pointed end of F-actin exposed to cytoplasm, and it is structurally protecting the network. ATP extraction of GFP-myosin VI from permeabilized cysts demonstrated that the myosin VI motor domain is important for localization. FRAP of GFP-myosin VI demonstrated that myosin VI remained bound to the actin cone for several minutes suggesting its role is structurally tethering or cross linking. These data suggest that myosin VI structurally stabilizing actin cone rather than transporting membrane vesicle.

189

**The PDZ Adaptor Protein Synectin Targets Myosin VI to Endocytic Vesicles**

A. Horowitz,[1] S. Naccache,[2] T. Hasson[2]; [1]Angiogenesis Research Center, Dartmouth Medical School, Lebanon, NH, [2]Division of Biological Sciences, University of California at San Diego, La Jolla, CA

We have recently shown that translocation of uncoated endocytic vesicles (UCV) through the cortical actin mesh is facilitated by myosin VI (myo6). This unconventional myosin is capable of supporting inward movement of UCV being the only known retrograde-moving actin-based molecular motor. The PDZ domain-containing adaptor protein synectin collocates with myo6 on UCV and may conceivably mediate myo6 recruitment to this organelle. We tested this hypothesis first by examining synectin targeting to UCV in myo6-null cells. Synectin was still localized to UCV, thus indicating that its targeting preceded and was not dependent on myo6. We next investigated the docking mechanism of synectin and myo6 to UCV by expressing a series of synectin mutants where either the N-terminus domain, the central PDZ domain, or the C-terminus domain was partially or completely truncated. A mutant consisting of the N-terminus and PDZ domains was the minimal fragment sufficient for synectin targeting to UCV. The myosin-VI binding motif was narrowed down to the carboxy terminus of synectin. Strikingly, a loss-of-function point mutation in the PDZ domain of synectin not only blocked UCV targeting, but also abolished myo6 binding. Saturation binding assays revealed that ligand binding to the PDZ domain of synectin increased the Vmax of the synectin-myo6 interaction, indicating that ligand binding has an allosteric effect - it stabilizes a synectin conformation where the myo6 binding site is accessible. The emerging docking mechanism

of myo6 to UCV suggests that the PDZ domain of synectin plays an active role in recruiting myo6 to UCV. The docking consists of two steps: (1) clathrin uncoating exposes the PDZ-binding motifs present in the carboxy-termini of transmembrane receptors, thus facilitating synectin binding; (2) once the PDZ domain is engaged, the myo6 binding motif in the carboxy-terminus becomes functional.

190

**TalinA is Stabilized by Binding to Myosin VII**

S. A. Galdeen, S. Stephens, M. A. Titus; Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN

Myosin VII (M7) and talin each function in both *Dictyostelium* and higher eukaryotes as a component of an adhesion complex that links membrane receptors to the actin cytoskeleton. Recent studies have identified talinA as a direct interactor with *Dictyostelium* M7 (DdM7) (Tuxworth, et. al. 2005) and suggested that this association is required for proper assembly of a membrane-receptor complex. In a separate study, Gebbie et. al. (2004) reported that talinA is absent in DdM7 null cells. The potential link between DdM7 and talinA levels has been examined in more detail to better understand the functional relationship between these two adhesion proteins. Western blotting reveals that talinA protein levels are linked to the expression of DdM7, with DdM7 null cells expressing 17% of the wild-type level of talinA. Real-time QPCR of cDNA from cells with altered DdM7 expression indicates that there is no change in talinA RNA levels, suggesting that DdM7 provides steric protection against talinA degradation. Examination of protein degradation kinetics confirms that talinA protein in cells lacking DdM7 is lost twice as fast as in wild-type cells. Interestingly, talinA localization is unaffected by the loss of DdM7. Overexpression of the DdM7 tail domain alone in DdM7 null cells restores talinA levels; however, the phagocytic defect that DdM7 null cells exhibit is not rescued, confirming that DdM7 has a function in adhesion that is independent of its binding to talinA. These results establish that DdM7 plays a role in function of an adhesion complex and stabilization of talinA *in vivo*.

191

**ATPase Cycle Mechanism of *Drosophila* Myosin VIIA**

S. Watanabe, R. Ikebe, M. Ikebe; Physiology, University of Massachusetts Medical School, Worcester, MA

Mutations of myosin VIIA cause deafness in species from human and mice to zebrafish and *Drosophila*. To understand the motor properties of myosin VIIA, we analyzed the kinetic mechanism of *Drosophila* myosin VIIA ATPase cycle using a truncated construct with entire neck domain (DM7AIQ5). $V_{max}$ and $K_{ATPase}$ of the steady state actin-activated ATPase activity of DM7AIQ5 were 1.2 $s^{-1}$ and 4.2 μM, respectively. The ATP hydrolysis rate (13.7 $s^{-1}$) was slow, but much faster than the $V_{max}$. The phosphate release rate from actoDM7AIQ5 (>50 $s^{-1}$) was also much faster than the $V_{max}$. Thus, ATP hydrolysis and phosphate release are not the rate-limiting steps. The rate of ADP release from actoDM7AIQ5 was 1.8 $s^{-1}$, which was close to the $V_{max}$. Pi-burst size was low (0.35 mol/mol) indicating that the equilibrium in ATP hydrolysis step is significantly shifted toward the pre-hydrolysis intermediate. Overall, the ATPase cycle rate of myosin VIIA is limited by slow ADP release. From the obtained data, it was concluded that myosin VIIA is a high duty ratio motor.

192

**Myosin-X (Myo10) is a Filopodial Motor Protein that Promotes Filopodia**

A. Bhaskar, B. W. Robertson, R. E. Cheney; Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Little is known about the molecular machinery regulating filopodia formation. Since Myo10 is a motor protein that localizes to filopodial tips, we sought to determine if Myo10 is a component of this machinery and have used scanning EM to ask if Myo10 promotes the formation of dorsal filopodia. We find that over-expressing Myo10 in cells such as COS-7 (which normally lack filopodia) dramatically increases dorsal filopodia. The MyTH4, but not the FERM domain of Myo10 is necessary for this filopodia promoting activity. Since VASP can also induce filopodia, we next tested if Myo10 can induce filopodia independent of VASP. Surprisingly, over-expression of Myo10 in Ena/VASP null MVD7 cells dramatically increases dorsal filopodia, demonstrating that Myo10 can induce filopodia in the absence of VASP. To test if endogenous Myo10 is necessary for filopodia formation, HeLa cells (which normally have numerous filopodia) were treated with a siRNA against Myo10. This treatment resulted in greater than 90% knockdown and a striking reduction in dorsal filopodia. To confirm that Myo10 is central to filopodia formation, we generated a coiled-coil construct to use as a potential dominant negative. Expression of this construct in HeLa cells also potently suppressed dorsal filopodia. Since Cdc42 is known to be a master regulator of filopodia formation, we next asked if Myo10 acts upstream or downstream of Cdc42. Myo10 siRNA treated HeLa cells transfected with constitutively active Cdc42 (which can induce filopodia in COS-7 cells) had remarkably reduced filopodia indicating that Myo10 is downstream of Cdc42. Taken together, these results demonstrate that Myo10 is a potent stimulator of filopodia, that the MyTH4 domain of Myo10 is necessary for filopodia formation, and that Myo10 functions downstream of Cdc42. Supported by an AHA fellowship to AB and NIH/NIDCD grant DC03299 to REC.

193

**Myosin X is a High Duty Ratio Motor**

K. Homma, M. Ikebe; Physiology, UMass Medical School, Worcester, MA

Myosin X is expressed in a variety of cell types and plays a role in cargo movement and filopodia extension, but its mechanoenzymatic characteristics are not fully understood. Here we analyzed the kinetic mechanism of the ATP hydrolysis cycle of acto-myosin X using a single-headed construct (M10IQ1). Myosin X was unique for the weak "strong actin binding state" (AMD) with $K_d$ of 1.6 μM due to the large dissociation rate constant (2.1 $s^{-1}$). $V_{max}$ and $K_{ATPase}$ of the actin-activated ATPase activity of M10IQ1 were 13.5 $s^{-1}$ and 17.4 μM, respectively. The ATP hydrolysis rate (>100 $s^{-1}$) and the phosphate release rate from acto-myosin X (>100 $s^{-1}$) were much faster than the entire ATPase cycle rate and, thus, not rate limiting. The ADP off rate from acto-myosin X was 23 $s^{-1}$, which was two times larger than the $V_{max}$. Pi-burst size was low (0.46 mol/mol) indicating that the equilibrium is significantly shifted toward the pre-hydrolysis intermediate. The steady-state ATPase rate can be explained by combination of the unfavorable equilibrium constant of the hydrolysis step and the relatively slow ADP off rate. The duty ratio calculated from our kinetic model, 0.6, was consistent with the duty ratio, 0.7, obtained from comparison of $K_{m\ ATPase}$ and $K_{m\ motility}$. Our results suggest that myosin X is a high duty ratio motor.

194

**Myosin Genes in Tetrahymena thermophila**

S. A. Williams, R. H. Gavin; Biology, Brooklyn College - CUNY, Brooklyn, NY

Analysis of the Tetrahymena thermophila macronuclear genome sequence revealed 13 myosin genes. This report presents an initial comparison of motor, neck, and tail domains of the 13 Tetrahymena myosins. The myosins in Tetrahymena did not align with any of the previously named myosin classes. Twelve of the myosins form a new class designated as XX. The other myosin is divergent from the twelve. Eleven of the motor domains in Tetrahymena myosins are slightly larger than the muscle myosin motor. Six of the Tetrahymena myosins contain putative IQ sequences. One of these myosins, Myo1, contains a putative IQ sequence within the FERM motif. The average length of the putative IQ sequence in the six Tetrahymena myosins is 22 aa. Seven of the Tetrahymena myosins do not contain an apparent IQ motif. Eleven of the myosins in Tetrahymena contain one or more coiled-coil sequences. The average number of heptads is 6, and only one of the Tetrahymena myosins, Myo13, appears to contain a sufficient number of heptads to support dimer formation. Seven of the Tetrahymena myosins do not contain known conserved tail domain motifs that could interact with cargo. Six of the myosins contain the myosin tail homology (MyTH4) and/or ezrin, radixin, moesin homology (FERM) motifs. One myosin contains only MyTH4 in the tail domain, and one myosin contains only FERM. Four of the Tetrahymena myosins contain both MyTH4 and FERM. Surprisingly, none of the myosins in Tetrahymena aligned with either class I, class II, or class V myosins. Apparent absence of a class II myosin is an indication that contractile ring assembly and function during cytokinesis in Tetrahymena either utilizes an unconventional myosin or does not require a myosin motor. [Supported by National Science Foundation grant 0130624]

195

**Processivity of Plant (*Chara*) Myosin**

K. Ito, E. Okutsu, Y. Wanikawa, Y. Hachikubo, K. Yamamoto; Biology, Chiba University, Chiba, Japan

Very fast cytoplasmic streaming (~70 μm/s) can be observed in fresh water alga *Chara*. This streaming is generated by the sliding movement of *Chara* myosin along the fixed cortical actin bundles with its tail attached to endoplasmic reticulum net work. Since many kinds of myosin carrying cargo are processive motor, we examined the processivity of *Chara* myosin by both landing assay and kinetic analysis. Landing rate of actin filaments on the glass surface covered with *Chara* myosin was similar to that of skeletal muscle myosin. The actin activated ATPase activity of this myosin was very high (Vmax = 388 Pi/head/s at 25 °C). We calculated duty ratio by measuring the time constant of strongly bound state. Strongly bound state consists two states, ADP bound state and nucleotide free state. The time constants of the ADP bound state and nucleotide free state were 0.4 ms and 0.1 ms, respectively at 25 °C. Since the total cycle time of ATPase activity was 2.6 ms, duty ratio was calculated to be 0.19. These results suggested that *Chara* myosin is not a processive motor. In the cytoplasm of *Chara*, myosin molecules are attached to the network of endoplasmic reticulum membrane and cannot diffuse away from actin bundles easily; the situation relative to actin is similar to that in skeletal muscle. Therefore, *Chara* myosin does not have to be a processive motor to generate cytoplasmic streaming.

196

**Interaction of *Chara* Myosin with Phospholipid Vesicles**

S. Nunokawa,[1] K. Shimada,[2] K. Ito,[1] K. Yamamoto[1]; [1]Biology, Chiba University, Chiba, Japan, [2]Kazusa DNA Institute, Chiba, Japan

Class XI unconventional myosin generates cytoplasmic streaming by moving along the fixed cortical actin bundles with its tail attached to endoplasmic reticulum membrane in characean alga. To explore the nature of the interaction with membrane, we expressed the globular tail domain of *Chara* myosin in insect cells, purified it, and studied its interaction with artificial phospholipid vesicles. It was found that the globular tail domain co-precipitated with vesicles made from acidic phospholipid such as phosphatidyl serine (PS) but not with ones made from neutral phospholipid such as phosphatidyl choline (PC). This interaction was weakened by the increase in the ionic strength suggesting that the interaction is electrostatic by nature. We then studied the affinity between the globular tail of *Chara* myosin and membrane vesicles made from phospholipid mixture to make the situation more natural. When the mixture contained 20 % PS and 80 % PC, the dissociation constant was 130 nM. We also measured the concentration of *Chara* myosin in the cell using quantitative immunoblot. We also calculated the amount of phospholipid in the endoplasmic reticulum of characean cells under certain assumptions. From these values, we estimated that more than 80 % of *Chara* myosin is attached to membrane just through electrostatic interaction.

197

**The Novel Myosin Myo19 is a Putative Motor for Mitochondrial Movement**

O. A. Quintero,[1,2] M. B. Kortan,[2] R. E. Cheney[1]; [1]Cell and Molecular Physiology, UNC-Chapel Hill, Chapel Hill, NC, [2]Biology, Franklin and Marshall College, Lancaster, PA

Mitochondria play key roles in ATP production, apoptosis, and calcium signaling. Although mitochondria are implicated in diabetes, aging, and neurodegenerative disorders, the mechanisms of mitochondrial movement and cellular localization remain unclear. Previous studies have shown that mitochondria move on both actin and microtubules in neuronal cells (RL Morris and PJ Hollenbeck, JCB, 1995). Here we report that a novel human myosin, tentatively named Myo19, localizes to mitochondria. Myo19 transcripts of approximately 4.3kb were identified in multiple tissues. Human Myo19 is a 970 amino acid protein consisting of a myosin motor domain, three IQ motifs, and a short tail region with no obvious homologies. The c-terminal 186 amino acids of Myo19 are necessary and sufficient for the localization of Myo19 to mitochondria, as GFP constructs lacking the tail region of Myo19 fail to localize to mitochondria, and GFP constructs consisting of just the c-terminal tail region localize to mitochondria. Overexpression of GFP-Myo19 tail in A549 cells resulted in a 50% decrease in average velocity of moving mitochondria compared to mitochondria in untransfected cells. In cells overexpressing full length GFP-Myo19, mitochondrial were observed with an asymmetric, “tadpole” shape with the wider end leading the movements. Mitochondria were observed to move for many micrometers at an average velocity of 81.5±4.2 nm/s. Treatment of A549 cells overexpressing full length GFP-Myo19 with 15μM nocodazole failed to abolish mitochondrial movements, whereas treatment with 500 nM latrunculin B resulted in the cessation of mitochondrial movements. Taken together, these results suggest that Myo19 is an unconventional myosin involved in the motility and localization of mitochondria. Supported by a SPIRE postdoctoral training grant NIH/GM00678 to OAQ, and NIH/NIDCD DC03299 to REC.

## Dynein I (198-211)

198

### Two-dimensional Averaged Images of the Dynactin Complex Revealed by Single Particle Analysis

H. Imai,[1,2] A. Narita,[1,2] T. A. Schroer,[3] Y. Maeda[1,2,4]; [1]Explatory Research for Advanced Technology (ERATO), Japan Science and Technology Agency (JST), Hyogo, Japan, [2]Structural Biochemistry Laboratory, RIKEN Harima Institute at SPring-8, Hyogo, Japan, [3]Department of Biology, Johns Hopkins University, Baltimore, MD, [4]Department of Biological Science, Nagoya University, Nagoya, Japan

The dynactin complex interacts with dynein and numerous other proteins to provide for a wide range of subcellular transport functions. A detailed understanding of dynactin structure and subunit organization is likely to yield new insights regarding dynactin function. In the present study, we used single particle analysis to obtain two-dimensional averaged image of dynactin complex isolated from chick embryo brains visualized by negative stain EM. The averaged image was highly reminiscent of previously published deep etch electron micrographs. The overall length distribution was tightly clustered as ≈40 nm. Each image showed a prominent shoulder complex and the pointed-end complex and mass associated with the barbed-end of the Arp1 mini-filaments were prominent in contrast to the simple Arp1 polymer that was observed by single particle analysis of brain dynactin (Hodgkinson et al., 2005). The differences should not be accounted for by different sources, but by disrupted preparations which were used for their analysis.

199

### A New Quick Freeze-Deep Etch-Tomographic Analysis of the Flagellar Outer Dynein Arm Complex

P. Lupetti,[1] S. Lanzavecchia,[2] D. Mercati,[1] F. Cantele,[2] R. Dallai,[1] C. Mencarelli[1]; [1]Dept. of Evolutionary Biology, University of Siena, Siena, Italy, [2]Dept. of Structural Chemistry, University of Milano, Milano, Italy

We present here a 3D reconstruction of in situ axonemal outer dynein arms obtained by electron tomography applied to a series of tilted images collected from metal replicas of rapidly frozen, cryofractured, and metal replicated sperm axonemes of the gall-midge fly *Monarthropalpus flavus*. This peculiar axonemal model consists of several microtubular laminae that proved to be sufficiently planar to allow the visualization of many doublet microtutules with their dynein complexes within the same fracture face. This structural feature allowed us to recover a significant number of equivalent objects and to improve the signal to noise ratio of the dynein reconstruction by applying advanced averaging protocols. The 3D model we obtained showed the following interesting structural features: First, each dynein arm has two head domains that are almost parallel and are obliquely oriented with respect to the longitudinal axis of microtubules. The two heads are therefore positioned at different distances from the surface of the A-tubule. Second, each head domain consists of a series of globular subdomains that are positioned on the same plane. Third, a stalk domain originates as a conical region from the proximal head and ends with a small globular domain that contacts the B-tubule. Fourth, the stem region comprises several globular subdomains and presents two distinct points of anchorage to the surface of the A-tubule. Finally, and most importantly, contrary to what has been observed in isolated dynein molecules adsorbed to flat surfaces, the stalk and the stem domains are not in the same plane as the head. This implies that, the force generated during the power stroke, would only act along the longitudinal axis of microtubules if a substantial rearrangement occurred in the stalk-head orientation.

200

### A Role for the Mitotic Checkpoint Protein ZW10 as a Vesicular Membrane Anchor for Cytoplasmic Dynein

D. Varma, R. B. Vallee; Cell Biology and Pathobiology, Columbia University, New York, NY

Cytoplasmic dynein is involved in a multitude of interphase cellular functions, including organization of the Golgi apparatus, endosome and lysosome transport, and maintenance of the centrosome-centered array of interphase microtubules. ZW10 is a mitotic checkpoint protein that is the well-established anchor for cytoplasmic dynein at mitotic kinetochores [Starr *et. al., JCB, 142 (3), 1998*]. However, it is expressed throughout the cell cycle, suggesting a potential interphase role as well. We have previously reported that ZW10 localizes to the Golgi apparatus in interphase cells and cosediments with Golgi membranes [Dujardin *et. al., Mol. Biol. Cell 12:163a*]. Furthermore we found that perturbation of ZW10 function by dominant negative overexpression and antibody injection caused dispersal of Golgi stacks, suggesting a role for ZW10 in interphase dynein function. Recently, however, ZW10 was reported to associate with a complex potentially involved in ER/Golgi trafficking, consisting of the t-SNARE syntaxin-18 and associated proteins, and the rad50-interacting protein RINT-1 [Hirose *et. al., EMBO J, 23 (6) 2004*]. The current study was undertaken to test directly for a role for ZW10 in interphase dynein function. Golgi, endosome, and lysosome markers were all disrupted by ZW10 RNAi. Microtubule organization was also disrupted in 80% of the cells, an effect associated with a substantial increase in centrosome number. Live-cell imaging in the subset of cells with normal radially arrayed microtubules revealed an ~70% decrease in the frequency of minus-end directed Golgi vesicular movements. Similar results were obtained with endosomal and lysosomal markers. Together, our results support a direct role for ZW10 in interphase dynein-mediated transport. Based on its role at the kinetochore, we propose that ZW10 may represent a novel and general interphase cargo anchor for dynein. Supp. by GM47434 to RBV

201

### Dynein Light Intermediate Chains: A Comparison of Distribution and Function in Fibroblasts and Developing Neurons

S. C. Tan, J. Tsai, G. Seale, R. B. Vallee; Department of Pathology, Columbia University, New York, NY

Cytoplasmic dynein is a multi-subunit molecular motor complex involved in retrograde vesicular organelle transport, cell migration, and mitosis. Dynein consists of the catalytic heavy chains, intermediate chains, light chains, and two light intermediate chains: LIC1 and LIC2. LIC1 was found to directly bind the centrosomal protein pericentrin (Purohit et al, 1999), indicating a role in mediating at least one form of dynein cargo-binding. The aim of this work is to determine the complete range of light intermediate chain functions as well as the individual and specific roles of LIC1 and LIC2. We have generated three LIC antibodies, which recognize respectively, LIC1, LIC2, and LIC1 and LIC2 together (Tynan et al, 2000). We examined and compared the subcellular localization of LIC1 and LIC2 within both fibroblasts and embryonic rat dissociated neurons and found LIC1 and LIC2 to have both unique and overlapping localization patterns. Distinct LIC1 and LIC2 puncta were seen in double-labeled dissociated neurons with LIC1 enriched at neurite tips while LIC2 was not. In fibroblasts, both LIC1 and LIC2 are localized to centrosomes and mitotic kinetochores. Overexpression of one LIC displaced the other from mitotic kinetochores. RNAi knockdown of LIC1 resulted in defective migration

of neural precursor cells while this process was unaffected by knockdown of LIC2. Together, these findings indicate that the LICs are not only important and necessary for certain dynein-dependent processes, but may have properties and roles specific to individual isoforms, allowing for more refined modulation of dynein function. Supp. by GM47434 to RBV.

202

**A Role for Huntingtin in Dynein/Dynactin-Mediated Vesicle Trafficking**

J. P. Caviston, S. M. Antony, M. K. Tokito, E. L. F. Holzbaur; Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA

Cytoplasmic dynein is a multi-subunit microtubule motor complex that, together with the dynein activator dynactin, actively drives vesicular cargo towards the minus-ends of microtubules. The mechanism by which dynein targets vesicular cargo is not fully understood. Htt is a vesicle-associated protein thought to play a role in vesicular transport. Polyglutamine repeat expansion in mutant htt causes the neurodegenerative disorder Huntingdon's Disease. However, htt is found in both neuronal and non-neuronal cells and loss of htt results in embryonic lethality in mice, suggesting that htt has an essential function. Mutant htt disrupts axonal trafficking in neurons, indicating that dynein-mediated trafficking may be compromised in the presence of mutant htt and huntingtin-associated protein 1 (HAP-1) interacts with dynactin, suggesting that htt and dynein/dynactin may function in similar pathways in the cell. We hypothesize that htt participates in cargo recruitment for dynein-mediated vesicle transport. In this study, we show that wild-type htt copurifies on vesicles with cytoplasmic dynein and dynactin. In a 2-hybrid screen, we identified Huntingtin (htt) as binding partner for dynein intermediate chain (DIC). By affinity chromatography, we detected an interaction between DIC and both *invitro* translated htt and htt derived from rat brain cytosol. Affinity-tagged htt associated with dynein and dynactin subunits purified from mouse and bovine brain. Immunocytochemistry revealed that htt colocalizes with microtubules in COS7 cells. Htt distribution overlaps with dynein and is concentrated in the Golgi complex. RNAi of htt resulted in Golgi disruption in a manner similar to the disruption of the Golgi that results when the dynein/dynactin interaction is compromised. Together, these findings implicate a role for htt in dynein/dynactin-mediated vesicle trafficking, possibly as an attachment protein that links vesicular cargo to dynein. Supported by NIH GM48661.

203

**Multiple Effects of LIS1 RNAi on Neural Progenitor Cell Division, Polarization, Aonogenesis, and Motility**

J. Tsai,[1] Y. Chen,[1] A. R. Kriegstein,[2] R. B. Vallee[1]; [1]Department of Pathology and Cell Biology, Columbia University, New York, NY, [2]Program in Developmental and Stem Cell Biology, UCSF, San Francisco, CA

Mutations in the human LIS1 gene cause classical lissencephaly (smooth brain), which has been proposed to result from neuronal migration defects. LIS1 functions in the cytoplasmic dynein pathway, but its cellular role is only partially understood. To explore the detailed mechanisms underlying this disease, we conducted the first *in situ* live-cell imaging analysis of LIS1-deficient neural progenitors throughout the entire radial migration pathway, which involves a series of polarization and repolarization steps. *In utero* electroporation of LIS1 shRNA-expressing cDNAs arrested cells at three morphological stages. The highly asymmetric radial glial cells from which neurons are generated retained their morphology, but interkinetic nuclear oscillations were arrested. Mitotic division in these cells, which occurs at the ventricular surface, was abolished, suggesting the polarized distribution of an unidentified mitotic signal. Most cells accumulated within the subventricular zone with a multipolar morphology. A single axon was produced as in normal cells, but its elongation and the conversion of the multiple processes to a single, thick dominant migratory process were abolished. Finally, the few cells that reached the intermediate zone exhibited normal bipolar morphology. However, somal translocation was arrested, while growth of the migratory process persisted. Together, these data reveal that LIS1 and, presumably, cytoplasmic dynein, direct a complex series of morphogenetic changes during neurogenesis, and have strikingly distinct roles in different process types. Also, despite the importance of LIS1 in this pathway, striking aspects of subcellular polarization persist in its absence. Our results appear to identify a novel mechanism by which nuclear position within radial glial progenitors controls their division. We are exploring this possibility further, and we are testing the role of other dynein regulatory proteins, such as NudE, in the neurogenesis pathway. *Supported by HD40182RBV, NS21223, NS35710 ARK.*

204

**A G59S Mutation in Dynactin That Causes Motor Neuron Degeneration Induces Protein Aggregation**

J. R. Levy,[1] C. J. Sumner,[2] M. K. Tokito,[1] K. E. Wallace,[1] K. H. Fischbeck,[2] E. L. F. Holzbaur[1]; [1]University of Pennsylvania, Philadelphia, PA, [2]NINDS, Bethesda, MD

Cytoplasmic dynein is the major microtubule minus end-directed motor in the cell. Dynactin is a required activator for dynein-driven processes, including vesicular transport, organelle localization, and mitotic spindle assembly. A G59S missense mutation in the human gene encoding the $p150^{Glued}$ subunit of dynactin results in an autosomal dominant, slowly progressive motor neuron disease. In this study, we examined the biochemical and cellular effects of this mutation. While the mutation affects microtubule binding, it also induces aggregation of $p150^{Glued}$. Sucrose density gradient centrifugation of cell lysates expressing mutant protein shows $p150^{Glued}$ in the 19S fraction, as expected due to the high mass of the dynactin complex. However, the mutant protein reveals a shift to higher density fractions as well, consistent with aggregation leading to heavier complexes. In addition, coimmunoprecipitation of differentially tagged constructs expressed *in vitro* demonstrate aggregation of the G59S polypeptide but not wild type $p150^{Glued}$. Immunofluorescence analysis of cells expressing the G59S mutation indicates the formation of both large, perinuclear, as well as small, cytoplasmically dispersed aggregates. Both dynein and dynactin are recruited to these aggregates; large aggregates also sequester mitochondria. Higher resolution imaging by electron microscopy reveals that the aggregates have a homogenous granular quality and are not membrane-bound. These data are consistent with the disease pathology, as immunohistochemical studies show cytoplasmic dynein- and dynactin-containing inclusions in motor neurons of brainstem tissue from an affected individual. Overexpression of the molecular chaperone Hsp70 inhibits the formation of aggregates in cells expressing the mutant protein, consistent with the hypothesis that the aggregates result from misfolding of the mutant protein and that enhanced aggregation of the mutant proteins leads to motor neuron degeneration in individuals expressing the G59S mutation of $p150^{Glued}$. Supported by NIH GM48661 and ALSA.

205

## Regulation of Cytoplasmic Dynein ATPase by Lis1

M. T. Mesngon,[1] C. Tarricone,[2] S. Hebbar,[1] A. M. Guillotte,[1] E. Schmitt,[3] L. Lanier,[4] A. Musacchio,[2] S. J. King,[3] D. S. Smith[1]; [1]Biological Sciences, University of South Carolina, Columbia, SC, [2]Department of Experimental Oncology, European Institute of Oncology, Milan, Italy, [3]Division of Mol. Biology and Biochemistry, University of Missouri-Kansas City, Kansas City, MO, [4]Department of Neuroscience, University of Minnesota, Minneapolis, MN

Mutations in Lis1 cause classical lissencephaly, a developmental brain abnormality characterized by defects in neuronal positioning. Over the last decade, a clear link has been forged between Lis1 and the microtubule motor cytoplasmic dynein. Substantial evidence indicates that Lis1 functions in a highly conserved pathway with dynein to regulate neuronal migration and other motile events. Lis1 interacts directly with dynein, but the mechanistic significance of this interaction is not well understood. We now report that recombinant Lis1 increases the microtubule-stimulated enzymatic activity of native brain dynein in vitro. To our knowledge, this is the first indication that Lis1 or any other factor directly modulates the enzymatic activity of cytoplasmic dynein. Binding and colocalization studies indicate that Lis1 associates with only a minor subset of dynein complexes. We propose a model in which the interaction between Lis1 and dynein stimulates a significant increase in the activity of individual motors. This would be particularly useful in neurons, which place high demands on motors for long distance axonal transport.

206

## CLIP-170 Homolog and NUDE Play Overlapping Roles in NUDF Localization in *Aspergillus nidulans*

V. P. Efimov,[1] J. Zhang,[2] X. Xiang[2]; [1]UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, [2]USUHS, Bethesda, MD

Several proteins involved in cytoplasmic dynein functions track the plus ends of microtubules when observed in live cells (reviewed by Carvalho *et al*., 2003). Among them, NUDF of *Aspergillus nidulans* (Lis1/Pac1) is a key component of the dynein pathway (Han *et al*., 2001). How NUDF is targeted to the microtubule plus end was unclear. Both CLIP-170 and NUDE physically interact with NUDF or its homologs and therefore are potential candidates for NUDF-targeting proteins (Efimov and Morris, 2000; Coquelle *et al*., 2002; Sheeman *et al*., 2003; Lansbergen *et al*., 2004). Here we investigate the *A. nidulans* homolog of CLIP-170, CLIPA. CLIPA has one CAP-Gly domain at the N-terminus and two zinc finger motifs at the C-terminus. GFP-CLIPA was observed in moving comets, representing its association with the growing microtubule plus ends. This localization was not dependent upon NUDA (dynein heavy chain), NUDF or the KipA kinesin (homolog of Kip2/tea2p) (Konzack *et al*., 2005). The *clipA* deletion mutant exhibited only a mild growth defect, and unlike the situation in yeasts (Berlin *et al*., 1990; Brunner and Nurse, 2000), cytoplasmic microtubules did not appear to be abnormally short and instead they clearly reached the hyphal tip. Combined with the *nudE* deletion mutant that also showed a mild growth phenotype, the double mutant exhibited a more dramatic growth defect. Deletion of both *clipA* and *nudE* almost completely abolished NUDF comets at the hyphal tip, while this dramatic effect was not observed in either single deletion mutant grown under the same condition. Thus, CLIPA and NUDE play overlapping roles in NUDF's plus-end targeting. Dynein comets were clearly present in the absence of both CLIPA and NUDE, demonstrating that different mechanisms are used for targeting dynein and NUDF in *A. nidulans*.

207

## The Role of NudE and NudEL in Mitotic Progression

S. A. Stehman, Y. Chen, R. B. Vallee; Pathology, Columbia University, New York, NY

NudE and NudEL are two closely related dynein regulatory proteins that interact with both the dynein complex and LIS1, a distinct dynein regulator responsible for the brain developmental disease lissencephaly. In contrast to cortical disorganization caused by LIS1 deficiency, NudE knockout mice exhibit a microcephalic phenotype (Neuron 44:279). The extent to which these outcomes reflect defects in neural cell migration vs. division has been the subject of debate (Tsai, J-W., et al., J. Cell Biol., in press). The current study seeks to determine common and distinct functional properties of NudE, NudEL, and LIS1, with an emphasis on mitosis. In previous work we observed NudE/L at the leading edge of migrating fibroblasts, a known site of dynein and LIS1 localization (J. Cell Biol. 163:1205), and showed that interference with NudE/L function blocked directed cell migration (Mol. Biol. Cell 15:394a). We also found NudE and NudEL to localize to mitotic kinetochores prior to dynein and LIS1 and remain at these sites until early anaphase, well after dynein, dynactin, and LIS1 had moved to the spindle poles (Mol. Biol. Cell 15:394a). In further contrast to LIS1, dynein, and dynactin, NudE/NudEL were absent from the mitotic cell cortex. Recent work from our lab has sought to explore these differences to determine how NudE and NudEL function in mitotic progression. Mitotic cells were injected with an anti-NudE/L antibody found to specifically interfere with the interaction between NudE/L and dynein. 60% of injected cells exhibited a mitotic defect, with an average duration for prometaphase-metaphase of 41.6 min compared. 24 min for controls. Further analysis is underway to test in detail at which stages of mitosis NudE and NudEL function. Supp. by HD40182 to RBV.

208

## Regulated Microtubule-binding of P150$^{Glued}$ And Search-capture

M. T. Raycroft, P. S. Vaughan, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

Microtubule (MT) tip-tracking is a unique behavior thought to reflect the dynamic association of some search-capture candidates with MT plus-ends. Phosphorylation has been implicated as one mechanism of release, however other mechanisms are also under investigation. To explore novel aspects of this phosphorylation model, we have analyzed the lagging recruitment of kinases, the impact of p150$^{Glued}$ mutants in the MT- binding domain, and coordination of other tip-tracking proteins. Our lab has proposed that lagging recruitment of MAPs and MT-bound kinases is the basis of MT plus-end specificity in overexpression experiments using GFP-tagged p150$^{Glued}$ expression constructs. To test the MAP recruitment model, we co-transfected MAP4 with CLIP-170 or p150$^{Glued}$ to reveal possible differences in MT-binding kinetics. MAP4 decorated the MT lattice with reduced labeling at the MT-tip, suggesting that MAPs display some lag in recruitment to the MT wall when compared to tip-tracking proteins of interest (CLIP-170 or p150$^{Glued}$). The G59S mutation in the MT-binding domain of p150$^{Glued}$, which is linked to human late-onset motor neuron disease, was engineered into GFP-p150$^{Glued}$ fusion constructs and tested by live-cell imaging. Wild-type p150$^{Glued}$ normally tip-tracks, while the G59S mutant failed to label MTs. To further test the impact of this mutation, we co-transfected siRNA (for endogenous p150$^{Glued}$) and GFP-G59S-p150$^{Glued}$. Unlike the wild type protein, the mutant construct was not able to rescue normal dynein-driven transport, and it also inhibited mitotic progression. Live-imaging was used to visualize GFP-p150$^{Glued}$with mRFP-EB1 or mRFP-CLIP-170. Our assays indicate that EB1, p150$^{Glued}$ and

CLIP-170 tip-track on the same MTs, and combined data from these co-transfection experiments suggest that there is a hierarchy of loading at the MT-tip. The MT-binding of p150$^{Glued}$ is a dynamic, regulated process providing valuable insight into search-capture components and dynein-mediated transport. (Supported by NIH GM60560).

209

### Function Evidence and Regulation of Tctex-1 Mediated Apical Transport in Polarized Epithelial Cells and Mammalian Photoreceptors

T. Yeh, J. Chuang, C. Sung; Ophthalmogy, Dyson Vision Research Institute, Weill Medical College of Cornell University, New York, NY

Cytoplasmic dynein plays a crucial role for the microtubule-based membrane vesicle trafficking, but its regulatory mechanism remains largely unknown. Our previous in vitro studies suggested that Tctex-1, a light chain of cytoplasmic dynein, functionally interacted with the C-terminus of rhodopsin. Subsequent studies showed that Tctex-1 mediated dynein motor activity was involved in the apical translocation of rhodopsin in polarized MDCK cells. Here, using a recently developed retinal transfection method, we showed that rhodopsin was mislocalized in mouse photoreceptors in which Tctex-1 was suppressed by siRNA. These results provide in vivo evidence that Tctex-1 mediates the polarized outer segment targeting of rhodopsin in rod photoreceptors. In addition, we identified a potential phosphorylation site within the region where Tctex-1 previously mapped to interact with dynein intermediate chain (IC). In vitro binding assays showed that the phosphomimic Tctex-1 mutant (i.e. E mutant) bound to rhodopsin, but not IC. The non-phosphomimic Tctex-1 mutant (i.e. A mutant), liked the wild type Tctex-1, bound both IC and rhodopsin. We examined how rhodopsin was targeted in MDCK monolayers inducibly expressing either the wild-type, the E mutant, or the A mutant. Consistent to the previous report, rhodopsin was apically targeted in MDCK cells expressing wild-type Tctex-1. However, rhodopsin was mislocalized to the basolateral surfaces in MDCK cells expressing either the E mutant or the A mutant. The mislocalization was unlikely to be due to the tight junction leak because the transepithelial electric resistance, the paracellular permeability, and the distribution of several other apical (e.g., gp135) and basolateral (e.g., Na+/K+-ATPase) markers appeared to be normal in all these cell lines. Thus, we propose that the dissociation between Tctex-1 and IC plays an important role in regulating dynein-mediated apical targeting in polarized epithelial cells.

210

### Design of Biomolecular Adaptors for retrograde Transport (BART) for the Transport of Novel Cargo by Dynein

R. N. Cohen,[1] M. J. Rashkin,[2] F. C. Szoka[1,2]; [1]Joint Graduate Group in Bioengineering, UC San Francisco/UC Berkeley, San Francisco, CA, [2]Biopharmaceutical Sciences, UC San Francisco, San Francisco, CA

The cytoplasm of a eukaryotic cell is a crowded, viscous milieu consisting of proteins, organelles, and RNA, which limit the diffusion of macromolecules. In order to generate and maintain intracellular organization, cells have evolved a system of molecular motors capable of moving large cellular components (cargo) from place to place along molecular filaments. For long distance transport, cytoplasmic dynein carries its cargo towards the microtubule minus end in the cell interior (retrograde) and kinesin carries its cargo towards the microtubule plus end in the cell periphery (anterograde). Our expanding knowledge of how these motors function and how they bind to their respective cargos makes it increasingly feasible to utilize the intracellular transport system for novel applications. We are attempting to target macromolecules or particles to dynein to increase the intracellular mobility of and the retrograde transport of novel cargos. A primary application for this technology is the delivery of DNA towards the nucleus for gene therapy. We have synthesized a small (22 amino acids) adaptor peptide that binds to the light chain 8 (LC8) of dynein to mediate the attachment of novel cargo to dynein. We designate this peptide a Biomolecular Adaptor for Retrograde Transport, or BART. Binding of LC8 to this peptide but not to a similar peptide with just 2 amino acids mutated has been confirmed by an affinity pull-down assay. A BART peptide that binds instead to a different dynein light chain, tctex1, is currently being designed. These peptides will be used in transport and particle tracking studies to determine if they can target conjugated cargo to dynein for transport to the cell interior.

211

### The Role of Cytoplasmic Dynein in Adenovirus Transport

K. H. Bremner, R. B. Vallee; Pathology, Columbia University, New York, NY

The minus end-directed microtubule motor cytoplasmic dynein transports a range of subcellular cargoes. It has also has been implicated in the translocation of various viruses to the nucleus, including adenovirus, a commonly used gene delivery vector with a relatively simple capsid composition [JCB 144:657-72 1999; Hum. Gene Ther. 11:151-65 2000]. The aim of the present study was to examine the molecular basis of the adenovirus 5-dynein interaction and therefore further our understanding of the mechanism and regulation of dynein cargo binding. Post-endocytic virus in synchronously infected cells was imaged by confocal microscopy at 60 minutes post-infection. We observe colocalization of the dynein heavy chain and light intermediate chains, as well as the dynactin p150$^{Glued}$ and Arp1 subunits, with incoming adenovirus capsids, including those bound to the nuclear envelope. We find that dynein, though not dynactin, co-immunoprecipitates with physiologically processed adenovirus particles recovered from infected cells, suggesting distinct roles for dynein and dynactin in adenovirus transport. We also find that the novel dynein regulatory proteins NudE/NudEL and NudC exhibit striking colocalization with incoming adenovirus, whereas LIS1 and ZW10 do not. Together, our data provide evidence for extensive utilization of the host cell dynein pathway by adenovirus. Supported by NIH grant GM47434 (RV) and an AHA postdoctoral fellowship (KB).

## Microtubule-Associated Proteins (212-237)

212

### Molecular Mechanisms of Microtubule Plus End Tracking Proteins

K. C. Slep,[1,2] R. D. Vale[1,2]; [1]Cellular & Molecular Pharmacology, University of California San Francisco, San Francisco, CA, [2]Howard Hughes Medical Institute, University of California San Francisco, CA

The Xmap215, Clip-170 and EB1 protein families represent distinct classes of microtubule associated protein that track along the tips of growing microtubules and that control microtubule dynamics, and mitotic spindle structure. To gain a better mechanistic understanding of how these proteins work, we have solved the crystal structures of the putative tubulin binding domains of Xmap215, Clip-170 and EB1. These atomic structures reveal distinct and unrelated protein folds, but in each case, amino acid conservation maps and mutagenic analyses reveal a conserved

face that appears to be responsible for tubulin association. To better understand how these domains operate, we have performed in vitro tubulin binding and nucleation assays and in vivo expression of GFP fusion proteins. Our results indicate that all of the proteins act as functional dimers. Single domains do not promote nucleation or associate with microtubules in vivo. However, dimerization confers both of these activities. This is dramatically demonstrated by chemical-induced dimerization. Monomeric EB1 or Clip-170 tubulin binding domains fused to FKBP and FRB are diffuse in the cytoplasm, but rapamycin-induced FKBP-FRB dimerization causes rapid relocalization to microtubule plus ends. Surprisingly, even chemical-induced heterodimerization of the structurally unrelated EB1 and Clip-170 binding domains results in microtubule plus end tracking. We propose that a unifying feature of these structurally diverse microtubule plus end tracking proteins is their ability to bind multiple tubulin subunits and deliver them to the plus end of microtubules, thereby allowing them to act as “chaperones” for microtubule polymerization.

213

### Potential APC and EB1 Functions in Scattering MDCK Cells

E. E. Morrison, C. Griffin, K. J. Langford, T. Lee, J. M. Askham; CRUK Clinical Centre at Leeds, Leeds, United Kingdom

The adenomatous polyposis coli (APC) tumour suppressor protein has been suggested to play a role in cell migration by capturing and stabilising microtubules at extending membranes and/or participating in centrosome reorientation towards the cell leading edge in a GSK3beta-dependent manner. We have correlated polarity development and cell movement with APC distribution in MDCKII epithelial cells scattering in response to PMA. Migratory polarity arose from the formation of a broad leading edge lamellipodium and rear retraction. The centrosome and Golgi apparatus were located towards the rear of migrating cells and stable microtubules were concentrated in retraction tails. Immunostaining indicated that APC clustered at the tips of retraction tails and the lateral margins of leading edge lamellipodia. This was confirmed by live imaging of cells expressing a GFP-APC fusion protein. Furthermore, live imaging of cells stably expressing EB1-GFP, an APC ligand, revealed the existence of novel, relatively immobile EB1-GFP structures at the cell periphery in both control and scattering cells that co-localised with APC in fixed cells. These structures may represent sites of active linkage between APC, EB1 and microtubule ends. Although closely juxtaposed, APC cortical clusters did not co-localise with focal adhesions but they defined cortical sites resistant to Latrunculin A-induced peripheral retraction. APC peripheral clusters were larger in cells expressing EB1-GFP than in control cells and cells expressing EB1-GFP spread more quickly during the initial stages of the scattering response. However, neither effect was seen in cells stably expressing potential dominant-negative EB1 mutants. Finally, GSK3beta inhibition antagonised APC clustering and morphological polarisation in PMA-treated cells. We propose that APC clusters link microtubules, via EB1, to stable, adhesive regions of the cell cortex that resist retraction during PMA-induced epithelial cell scattering.

214

### Structural Basis for the Activation of Microtubule Assembly by the EB1 and p150$^{Glued}$ Complex

I. Hayashi,[1] A. Wilde,[2] T. K. Mal,[1] M. Ikura[1]; [1]Department of Medical Biophysics, University of Toronto, Ontario Cancer Institute, Toronto, ON, Canada, [2]Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada

Plus-end tracking proteins, such as EB1 and the dynein/dynactin complex, regulate microtubule dynamics. These proteins are thought to stabilize microtubules by forming a plus-end complex at microtubule growing ends with ill-defined mechanisms. Here we report the crystal structure of two plus-end complex components, the carboxy-terminal dimerization domain of EB1 and the microtubule-binding (CAP-Gly) domain of the dynactin subunit p150$^{Glued}$. Each molecule of the EB1 dimer contains two helices forming a conserved four-helix bundle, while also providing p150$^{Glued}$-binding sites in its flexible tail region. Combining crystallography, NMR and mutational analyses, our studies reveal the critical interacting elements of both EB1 and p150$^{Glued}$, whose mutation alters microtubule polymerization activity. Moreover, removal of the key flexible tail from EB1 activates microtubule assembly by EB1 alone, suggesting that the flexible tail negatively regulates EB1 activity. We, therefore, propose that EB1 possesses an autoinhibited conformation which is relieved by p150$^{Glued}$ as an allosteric activator.

215

### The Microtubule Plus End-Binding Protein EB3 is Essential for Muscle Cell Differentiation

A. Straube,[1] A. Merdes[2]; [1]Wellcome Trust Centre for Cell Biology, University of Edinburgh, United Kingdom, [2]CNRS - Pierre Fabre, Toulouse, France

Upon muscle cell differentiation, myoblasts elongate and fuse at their cell poles with each other to form large multinuclear myotubes. This process involves a striking reorganization of the microtubule cytoskeleton. Microtubules align along the length of the cell while centrosomes disassemble and centrosomal proteins are recruited to the nuclear surface. We identified the microtubule plus end-binding protein EB3 as a key player in these processes. Although EB3 is expressed only at very low levels in undifferentiated myoblasts, its depletion using specific shRNAi constructs results in many curly microtubules. In contrast, depletion of the abundant and closely related protein EB1 has no obvious effects on the microtubule organization. However, depletion of both EB1 and EB3 enhances the curly microtubule phenotype, indicating that EB1 can partially rescue the loss of EB3. EB3 is upregulated early during muscle differentiation, suggesting that it fulfils an important role during cell polarisation and microtubule reorganization. Indeed, depletion of EB3 prevents myoblast fusion. EB3 depleted cells show defects in bipolar cell elongation and microtubule organization. As cell migration was not compromised we speculate that microtubule-dependent signalling at the cell poles might be affected in EB3-depleted cells. Experiments are under way to examine the mechanisms by which EB3 helps to accomplish muscle cell differentiation.

216

### Mal3p, the *S pombe* Homolog of EB1, Localizes Preferentially to the Microtubule Lattice Seam

L. Sandblad,[1] K. E. Busch,[2] P. Tittmann,[3] H. Gross,[3] D. Brunner,[2] A. Hoenger[1]; [1]SBBC, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, [2]CBB, EMBL, Heidelberg, Germany, [3]Electron Microscopy Center ETH Zurich, Swiss Federal Institute of Technology, Zurich, Switzerland

*In vivo* dynamics and spatial organization of Microtubules (MTs) are regulated by highly conserved Microtubule-associated proteins (MAPs), e.g. end-binding protein1 (EB1). We are investigating the structure and function of MTs complexes with a subset of MT plus end interacting proteins (+TIPs), using *in vitro* assays and electron microscopy. The *S. pombe* protein Mal3p belongs to the EB1-family, and is a central component in the polarity determination mechanism. Mal3p, like its human homolog EB1, was previously reported to bind MT plus ends and after over expression

also to the MT lattice. With high-resolution surface shadowing electron microscopy we found Mal3 binding on the MT surface in an interesting manner, never demonstrated for any other MAPs before. Mal3p preferentially binds along one single groove in between two protofilaments. The binding sites repeat with 8 nm intervals, corresponding to the length of a αβ-tubulin dimer. Geometrical analyses of the Mal3p-MT interaction show a pattern according to a MT A-lattice, the tertiary conformation of the MT lattice seam. Our findings suggest that Mal3p regulates MT dynamics through a specific interaction with the MT lattice seam. Furthermore, we currently investigate the interaction between MTs, Mal3p and other +TIPs, to gain insight into the structure and function of these complexes. Tip1p, the *S. pombe* homolog of CLIP170 and the kinesin like motor protein Tea2p are both dependent on Mal3p for proper interaction with MTs and their transport to the growing plus end.

217

**The Role Of Plus-end-tracking Proteins (+tips) In Dynein-mediated Endosome Traffic**

J. H. Lenz, I. Schuchardt, G. Steinberg; Department of Organ. Interactions, Max Planck Institute of Terrestrial Microbiology, Marburg, Germany

In higher eukaryotes endosome motility is mediated by microtubule-dependent motors. We have previously shown that minus-end directed dynein participates in bidirectional traffic of early endosomes (EE) in yeast-like cells of the model fungus *Ustilago maydis*. Here we demonstrate that dynein supports retrograde long-distance transport of EE in the elongated hyphae. Most dynein localizes to the microtubule (MT) plus-ends in the hyphal tip. Life cell imaging revealed that EE migrate into this dynein accumulation before they reverse direction. Thus, the apical concentration might be a reservoir of inactive motors that become loaded with EE at MT plus-ends and become activated for minus-end directed organelle traffic. Consistent with this notion, in null mutant hyphae of *kin1*, a Kinesin-1 that targets dynein to the plus-ends in *U. maydis*, retrograde EE traffic is abolished. Furthermore, depletion of the putative dynein activator LIS1, which colocalizes with dynein at MT plus-ends, significantly increases the amount of dynein in the tip and abolished retrograde EE traffic. Surprisingly, neither EE motility nor dynamic instability of MTs was affected in mutants deleted in a CLIP-170 homologue. These findings contradict the assumed role of this +TIP in anchoring EE and dynein at the MT tip. Instead, our results indicate that dynein is anchored at the plus-ends by a yet unknown mechanisms that might involve the dynactin complex. Further studies are underway that address the role and localization of dynactin in retrograde, dynein-dependent EE transport.

218

**Phosphorylation Regulates CLIP-170 Conformation and Kinetics**

H. Lee,[1] E. S. Nadezhdina,[2] Y. A. Komarova,[1] G. G. Borisy[1]; [1]Cell and Molecular Biology, Northwestern University, Chicago, IL, [2]Institute of Protein Research, Moscow, Russian Federation

Cytoplasmic Linker Protein 170 (CLIP-170) is a microtubule plus end-tracking protein which binds to growing microtubule plus ends. CLIP-170 conformation switches between "open" and "closed states via an intramolecular association between its $NH_2$-terminal head and COOH-terminal tail and regulates its binding to microtubules and dynactin (Lansbergen et al., JCB 2004). Here we show that the intramolecular association is regulated by phosphorylation. Addition of the third serine-rich region to the head domain of CLIP-170 diminished its ability to interact with the CLIP-170 tail in a co-immunoprecipitation assay. This effect was relieved by treatment of cells with the de-phosphorylation-inhibiting drug, okadaic acid, suggesting that phosphorylation of residues located in the third serine-rich region are important for stabilizing the CLIP-170 folded conformation. To determine the potential regulatory sites involved in the head-to-tail interaction, we performed in-vitro site-directed mutagenesis on 16 conservative serines located in the third serine-rich region, and analyzed head-tail interaction by domain co-immunoprecipitation assays. We identified four key serine residues: S311, S313, S319 and S320. Mutation of these residues to Ala which mimics the dephosphorylated state resulted in disruption of the head-to-tail interaction while mutation of the same residues to Glu which mimics the phosphorylated state stabilized this interaction. The ala mutants also induced more efficient binding of full length CLIP-170 to taxol-stabilized microtubules compared to the Glu mutant or wild-type CLIP-170 in a microtubule-pelleting assay suggesting that dephosphorylated CLIP-170 has higher affinity for the microtubule lattice. Consistent with this result, YFP-CLIP-170-Ala mutant dissociated twice slower from the growing plus ends of microtubules in vivo whereas YFP-CLIP-170-Glu dissociated twice faster than wild-type CLIP-170. We conclude that critical serine residues control CLIP-170 conformational transitions and their phosphorylations are essential in order to ensure stable interaction between the head and tail. Supported by NIH GM25062 to GGB.

219

**The Minimal Functional Unit for CLIP-170 Plus-End Tracking**

E. S. Folker, H. V. Goodson; Biochemistry, University of Notre Dame, Notre Dame, IN

Because the molecular events at the microtubule plus-end govern whether a microtubule will grow or shrink, the proteins that localize specifically to this region are likely to play a role in the regulation of the microtubule cytoskeleton. The answer to *how* these proteins localize to the plus-end will probably provide insight into the *why* these proteins track microtubule plus-ends. We have recently provided evidence that CLIP-170 uses a preassociation and copolymerization mechanism to track microtubule plus-ends. However yeast CLIP-170 orthologs track microtubule plus-ends by a kinesin dependent mechanism, suggesting that other mechanisms might contribute to CLIP-170 plus-end tracking behavior . To address this and other questions about the CLIP-170 +TIP behavior, truncations of CLIP-170 were made to separate the two conserved CAP-Gly domains. The abilities of each construct to track microtubule plus-ends, bind tubulin, bind microtubules, and bind EB1 were then tested. These analyses lead to the following conclusions. First, the ability of CLIP-170 to bind tubulin with high affinity is not sufficient for CLIP-170 to track microtubule plus-ends, and may not be required. However, tracking in the absence of a high affinity interaction with tubulin is rare, spatially constrained, and not obvious. Moreover, constructs that do bind tubulin with high affinity track microtubule plus-ends most robustly. Second, binding to EB1 likely contributes to, but is not sufficient for CLIP-170 +TIP behavior in the absence of a high affinity interaction with tubulin. Finally, the first CAP-Gly is required for CLIP-170 plus-end tracking behavior. These findings suggest that cellular conditions add complexity to the previously described preassociation and copolymerization mechanism for CLIP-170 plus-end tracking behavior.

220

**The CLIP170 Homologue Bik1p, the cyclin Clb5p, and Kar9p Phosphorylation in Yeast**

J. K. Moore, S. D'Silva, R. K. Miller; Department of Biology, University of Rochester, Rochester, NY

Accurate positioning of the mitotic spindle in *S. cerevisiae* is coordinated with the asymmetry of the two poles and requires the microtubule-to-actin linker, Kar9p. The asymmetric localization of Kar9p to one SPB and microtubule (MT) plus ends requires Cdc28p, the yeast Cdk1. Here, we show that the CLIP170 homologue, Bik1p, binds directly to Kar9p. In the absence of Bik1p, Kar9p localization is not restricted to the daughter-bound SPB, but is instead found on both SPBs. *bik1Δ* mutants also exhibit a defect in SPB inheritance that is proportional to the percentage of cells in which Kar9p localizes to both poles. Kar9p is hypo-phosphorylated in *bik1*Δ mutants and Bik1p binds to both phosphorylated and unphosphorylated isoforms of Kar9p. Furthermore, the two-hybrid interaction between full length *KAR9* and the cyclin *CLB5* requires *BIK1*. The binding site of Clb5p on Kar9p maps to a short region within the basic domain of Kar9p that that is adjacent to the binding site for Bik1p and contains a conserved phosphorylation site, serine 496. Mimicking phosphorylation at this residue with a S496E mutation restores Kar9p-GFP localization to one SPB in both *bik1*Δ and *clb5*Δ cells. Together, these data suggest that Bik1p promotes the phosphorylation of Kar9p by facilitating the Kar9p-Clb5p interaction, which in turn restricts Kar9p to one SPB and associated cytoplasmic microtubules. These findings provide further insight into a mechanism for directing centrosomal inheritance.

221

### Kar9p Interacts with Smt3p and the Machinery for Sumoylation in Yeast

N. Meednu,[1] H. Hoops,[2] L. Jablonski,[1] E. Sia,[1] R. K. Miller[1]; [1]Department of Biology, University of Rochester, Rochester, NY, [2]Department of Biology, State University of New York, Geneseo, NY

Kar9p plays an important role in positioning the mitotic spindle by serving as a linker between the actin and microtubule cytoskeletons. The microtubule-to-Kar9p linkage occurs through Bim1p, a microtubule binding protein and EB1 homologue. However, little is known about the molecular mechanisms that regulate Kar9p function. SUMO/Smt3p is a conserved small ubiquitin-related modifier that is covalently attached to other proteins as a post-translational modification. SUMO is analogous to ubiquitin in several ways, but functionally different. Unlike ubiquitin, sumoylation does not target proteins for degradation. Although the targets of sumoylation are associated with a diverse set of biological processes, it was not previously known whether sumoylation regulates the microtubule-dependent process of spindle positioning in yeast. Here, we show that Kar9p and Bim1p are likely to be modified by SUMO/Smt3p. Both interact with *SMT3* by two-hybrid analysis. However, neither interact with a mutant form of *SMT3* in which the terminal glycine required for conjugation has been mutated to alanine. Both *KAR9* and *BIM1* also interact with proteins involved in the sumoylation pathway-- *UBC9* an E2 enzyme required for SUMO conjution; *NFI1* an E3 enzyme conferring specificity for SUMO targets; and *WSS1* a weak suppressor of SUMO. The Kar9p-Ubc9p interaction was confirmed *in vitro* by affinity chromatography. A single point mutation in *KAR9*, *kar9*-L304P, disrupts the two-hybrid interaction with *SMT3*, *UBC9*, and *WSS1* but retains its interactions with other proteins important for spindle positioning, *BIM1*, *MYO2*, *BIK1*, and *STU2*. Phenotypic analysis of the *kar9*-L304P mutant demonstrates that its spindle is mis-positioned relative to the bud neck. Combined, these data suggest that sumoylation may be a new mechanism for the regulation of Kar9p and Bim1p function during spindle positioning in yeast.

222

### Phosphorylation of CLASP2γ by GSK-3β Regulates the Interaction with IQGAP1

J. Noritake,[1] T. Watanabe,[1] T. Harada,[1] T. Matsui,[1] S. Wang,[1] M. Fukata,[2] K. Kaibuchi[1]; [1]Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Laboratory of Genomics and Proteomics, National Institute for Longevity Science, Obu, Japan

Links between the plus-ends of microtubules and cortical regions are essential for the establishment of cell polarity and directional migration. The plus-end-tracking proteins (+Tips) accumulate at the plus-ends of growing microtubules and play pivotal roles in sensing cortical capture sites. CLIP-associating proteins (CLASPs), one of +Tips, are known to regulate the microtubule network cooperating with the cytoplasmic linker proteins (CLIPs) to maintain cellular asymmetry. Recent studies about CLASPs have suggested that Rac1 and GSK-3β regulate the CLASP-microtubule association. Here we found that CLASP2γ formed a quaternary complex with IQGAP1, GTP-bound form of Rac1/Cdc42, and CLIP-170, and colocalized with IQGAP1 at the leading edge of Vero fibroblasts, where specific subsets of microtubules are targeted. Our further analysis revealed that GSK-3β phosphorylated CLASP2γ and the phosphorylation of CLASP2γ inhibited its interaction with IQGAP1. On the other hand, the interaction of CLASP2γ with IQGAP1 was restored by the treatment of GSK-3β inhibitors such as Lithium Chloride and SB21673 in Vero fibroblasts. These results suggest that GSK-3β negatively regulates the interaction of CLASP2γ with IQGAP1, and CLASP2γ stabilizes microtubules at the cell periphery together with IQGAP1 and CLIP-170.

223

### Spatial Regulation of CLASP Affinity for Microtubules by Rac1 and GSK3ß in Migrating Epithelial Cells

T. Wittmann, C. M. Waterman-Storer; Cell Biology, The Scripps Research Institute, La Jolla, CA

Cell locomotion depends on polarization of the cytoskeleton including actin and microtubules. We previously observed that microtubule dynamic instability is locally regulated in migrating cells downstream of the small GTPase Rac1 (*J. Cell Biol.* **161**:845-851), a regulator of actin polymerization dynamics in migrating cells. Proteins that in cells specifically bind to growing microtubule plus ends (+TIPs) are thought to play important roles in polarization of the cytoskeleton. However, most +TIPs such as EB1 and CLIP-170 do not show a bias of their microtubule-binding behavior toward different subcellular regions. Here, we examine the dynamics of the +TIP CLASP (mammalian homologue of *Drosophila* orbit/mast) in migrating PtK1 epithelial cells (*J. Cell Biol.* **169**:929-939). We find that, although CLASPs track microtubule plus ends in the cell body, they decorate the entire microtubule lattice in the leading edge lamella. FRAP reveals fast turnover of CLASP on lamella microtubule lattices. Rapid recovery of CLASP fluorescence also occurred just behind the growing microtubule plus end in the cell body, which has implications for the CLASP plus end-loading mechanism. Microtubule lattice binding is mediated by the COOH-terminal region of the CLASP microtubule-binding domain, which is also required for high affinity binding to taxol-stabilized microtubules in vitro. Microtubule lattice binding is regulated downstream of Rac1 and glycogen synthase kinase 3β (GSK3β). Phosphorylation of sites in the N-terminal part of the CLASP microtubule-binding domain by GSK3β likely regulates the affinity of CLASPs for microtubule lattices. These results demonstrate the striking difference of the microtubule cytoskeleton in the lamella as compared with the cell body and provide the first direct observation of subcellular regulation of a microtubule-associated protein in migrating cells. These results also suggest a complex, local regulation of CLASP affinity for microtubules.

224

**CLAMP, A Novel Microtubule-Associated Protein with EB-Type Calponin Homology**

G. W. Dougherty,[1] H. J. Adler,[2] A. Rzadzinska,[3] M. Gimona,[1] Y. Tomita,[4] C. Lattig,[5] R. Merritt,[6] B. Kachar[6]; [1]Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Chieti, Italy, [2]Biology, University of Maryland, College Park, MD, [3]Genetics of Deafness, Wellcome Trust Sanger, Cambridge, United Kingdom, [4]Lombardi Cancer Center, Georgetown University, NW Washington, DC, [5]G-Protein Coupled Receptors, NINDS / NIH, Bethesda, MD, [6]Cell Structure, NIDCD / NIH, Bethesda, MD

Microtubules (MTs) are polymers of α and β tubulin dimers that mediate many cellular functions, including the establishment and maintenance of cell shape. The dynamic properties of MTs may be influenced by tubulin isotype, post-translational modifications of tubulin, and interaction with microtubule-associated proteins (MAPs). End-Binding (EB) family proteins affect MT dynamics by stabilizing MTs, and are the only MAPs reported that bind MTs via a calponin-homology (CH) domain (Bu and Su, 2003; Tirnauer and Bierer, 2000). Here we describe a novel 27 kDa protein identified from an inner ear organ of Corti library. Structural homology modeling demonstrates a CH domain in this protein similar to EB proteins. Northern and Western blotting confirmed expression of this gene in other tissues including brain, lung, and testis. In the organ of Corti, this protein localized throughout distinctively large and well-ordered MT bundles that support the elongated body of mechanically stiff pillar cells of the auditory sensory epithelium. When ectopically expressed in Cos-7 cells, this protein localized along cytoplasmic MTs, promoted MT bundling, and efficiently stabilized MTs against depolymerization in response to high concentration of nocodazole and cold temperature. We propose that this protein, designated CLAMP, is a novel MAP and represents a new member of the CH domain protein family.

225

***Adenomatous Polyposis Coli* is Required for Proper Cytokinesis by Affecting Anaphase Spindle Dynamics**

C. M. Caldwell, R. A. Green, K. B. Kaplan; University of California, Davis, Davis, CA

We have shown that mutations in the tumor suppressor gene *adenomatous polyposis coli* (e.g., $APC^{1-1450}$) dominantly inhibit microtubule dynamics and thus compromise chromosome alignment during metaphase (Green and Kaplan 2003, Green et al 2005). In addition, we have observed that expression of $APC^{1-1450}$ results in an accumulation of multinucleated cells. As the cytokinetic furrow has been shown to be specified by anaphase spindle microtubules, we hypothesized that APC mutants that affect spindle dynamics fail to properly regulate cortical activities required for cytokinesis. Consistent with this hypothesis, we observed cytokinetic defects in cells expressing $APC^{1-1450}$. One population of cells initiates anaphase but fails to complete cytokinesis, exiting mitosis and forming binucleate cells. A second population of cells was observed to delay furrow initiation, often forming asymmetric furrows before completing cell division. To address the cause of the cytokinetic defects, we examined the localization of the cytokinetic determinants, Aurora B, RhoA and actin. In cells expressing $APC^{1-1450}$ AuroraB fails to localize properly to the spindle midzone. Similarly, RhoA and actin are also mis-localized; instead of a polarized distribution, RhoA and actin are evenly distributed to the polar and medial cortex in cells expressing $APC^{1-1450}$. These patterns are consistent with the aberrant furrow formation observed and suggest that APC normally regulates the activity and/or position of cytokinetic determinants. Similar cytokinetic defects are observed when microtubule dynamics are inhibited using drug treatment or by inhibiting the plus-end binding protein, EB1. Together, these data suggest that APC modulates microtubule dynamics throughout mitosis and that multiple mitotic defects may contribute to the chromosome instability observed in colorectal tumor cells.

226

**GEF-H1, a Microtubule-Regulated Exchange Factor, Modulates Rho Function Required for Cytokinesis**

J. Birkenfeld, P. Nalbant, B. Bohl, G. M. Bokoch; Immunology, The Scripps Research Institute, La Jolla, CA

Formation of a functional actomyosin contractile ring (cleavage furrow; CF) during cytokinesis requires coordinated cycles of RhoA between the active GTP-bound state and the inactive GDP-bound form. Microtubules (MTs) may play an important role in induction and positioning of the CF by providing a stimulus for the localized activation of RhoA. In this study, we address the role of GEF-H1, a RhoA-specific nucleotide exchange factor that is regulated by MT binding in the localized RhoA activation during cytokinesis. First, we show that perturbation of endogenous GEF-H1 function by both, overexpression of inhibitory mutants and siRNA-mediated gene knock down led to the formation of multinucleated cells, a clear indication of failed cytokinesis. In addition, subcellular distribution of GEF-H1 correlated with the formation of the acto-mysosin CF. We found GEF-H1 immnuoreactivity to accumulate at regions of the mitotic apparatus known to be essential for cytokinesis such as the spindle midzone and the midbody. Furthermore, since GEF-H1 interacted with RhoA during CF formation and ingression (as demonstrated in GST-RhoAT19N pull down assays) and, GEF-H1 depletion caused a dramatic decrease in RhoA activation during telophase (as measured by RBD pull down assays) GEF-H1 enzymatic activity appears to be a crucial factor for localized RhoA activation during cytokinesis.

227

**Regulation of Microtubule Stability *in vitro* and in Transfected Cells by MAP1B and SCG10**

P. Bondallaz,[1] A. Barbier,[1] S. Soehrman,[1] G. Grenningloh,[2] B. M. Riederer[1,2]; [1]DBCM, University of Lausanne, Lausanne, Switzerland, [2]Psychiatric Neuroscience Center, University Hospital of Lausanne, Prilly, Switzerland

Microtubule stability and dynamics are regulated by a variety of proteins. Here, opposite effects on microtubule stability of two proteins found in the growth cone, MAP1B and SCG10, were examined by co-transfection of COS-7 cells and using recombinant proteins in microtubule assembly assays. Microtubule binding was evaluated by electron microscopy. In cells transfected with a 158kDa MAP1B fragment containing the microtubule-binding site, over 80% of the transfected cells maintained intact microtubules upon exposure to 3 µM colcemid. While the microtubule network disappeared in cells transfected with SCG10, a proportion of SCG10 and MAP1B double-transfected cells maintained intact microtubules, suggesting that MAP1B was able to protect microtubules from SCG10 induced dissassembly. However, cells with high expression levels of SCG10 contained little or no microtubules despite the presence of MAP1B. This indicates that SCG10 is more potent to destabilize microtubules than MAP1B to rescue them. In microtubule polymerization assays, MAP1B was able to promote tubulin assembly at a ratio of 1 MAP1B per 70 tubulin dimers and increased resistance of microtubules to the influence of destabilizing drugs. MAP1B also promoted microtubule polymerization at SCG10 concentrations lower than 8 µM. Above 10 µM SCG10, microtubule assembly was blocked independent of the presence of MAP1B. This supports the idea that SCG10 is a tubulin sequestering protein and that MAP1B needs free tubulin dimers to promote assembly. Furthermore, we

found that SCG10 can also bind along the microtubules, both in vitro and in vivo. These studies show that the balance between assembly and disassembly can be shifted by small differences in SCG10 concentration, indicating an important role of a critical balance between MAP1B and SCG10 for the regulation of microtubule dynamics in growth cones.

228

**MAP1B Interacts with Actin and is a Scaffold Protein**

N. Cueille,[1] S. Popa-Nita,[2] G. Dietler,[2] B. M. Riederer[1,3]; [1]DBCM, University of Lausanne, Lausanne, Switzerland, [2]Department of Physics, EPFL, Lausanne, Switzerland, [3]Psychiatric Neuroscience Center, CERY, Prilly, Switzerland

MAP1B is an essential protein during brain development and during neurite outgrowth. Here we have identified by a MAP1B pulldown assay, by 2D gels analysis and by mass spectrometry (MALDI-TOF) its interacting partners. Results were confirmed by Western blots in brain tissue and cell cultures. Furthermore, binding forces were determined by atomic force microscopy. Not surprisingly, major MAP1B associated proteins were tubulin and actin. Both co-precipitated throughout development at similar ratios. MAP1B-actin interaction does not seem to depend on the MAP1B phosphorylation state because actin was also precipitated from brain tissue after exposure to alkaline phosphatase. Especially in newborn tissue, another precipitatated protein of high stoichiometry was glyceraldehyde-3-phosphate dehydrogenase. At lower stoichiometry, a variety of proteins were identified such as heat shock protein 8, dihydropyrimidinase related protein 2, protein-L- isoaspartate O-methyltransferase, ß-spectrin, clathrin protein MKIAA0034 and g-actin. Such proteins seem associated to MAP1B either directly or indirectly via actin, tubulin or GPDH. Differences in association vary, depending on the development state or on the cell type used in the pulldown assay. Interacting forces between MAP1B itself and actin or tubulin were measured by atomic force microscopy and were between 100-180pN. Given the large number of proteins apparently associated with MAP1B, one can conclude that MAP1B has not only a microtubule-stabilization effect, but must act as an actin anchor and may play a role as a scaffold protein for a variety of proteins, this especially at the tip of growth cones or towards the cortical actin cytoskeleton. This work was supported by FNRS grant 3100-067201.01.

229

**Comparisons of Microtubule Binding Behavior for Several MAPs**

A. D. Stephens, D. S. Razafsky, S. J. King; School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO

We are interested in characterizing how different microtubule-associated proteins (MAPs) bind to microtubules. We have analyzed MAP binding domains using a novel single-particle binding assay. In the assay proteins were bound to polystyrene beads, the beads were added to a flow cell that had microtubules attached to the surface, and bead movements were recorded with a microscope as the protein bound to microtubules. Various particle tracking software were utilized to analyze bead movement. Mean-squared displacement (MSD) analysis was also used to quantify and classify different binding events by tracking a bead's displacement over time. Tau-coated beads bound to microtubules at a single spot and the beads exhibited short-range diffusion in a circular area from a fixed tether position. The CAP-Gly domain of p150 and the GMAD domain of Gas11 bound similar to Tau except for an increase in the length of the tether between the bead and microtubule. For the basic microtubule binding domain of p150 some beads behaved similar to that seen by the p150 CAP-Gly domain but in other beads the point at which the protein tether contacted the microtubule moved along the microtubule instead of being fixed. The p150 basic domain was the only microtubule binding domain that exhibited this "skating" behavior. The MSD analysis of these "skating" events showed an exponential relationship indicative of semi-constant displacement over time even without a motor protein present. The IMAD domain of Gas11 appeared to have an inhibitory effect on the GMAD/microtubule interaction. The IMAD domain caused the GMAD to have a larger and varying mean-squared displacement. Other MAPs will be analyzed to determine if they fall into one of the classes we observed or if they belong to one or more new classes of microtubule binding behavior.

230

**Role of MAP200, a Member of Plant XMAP215/Dis1 Family, in Tubulin Polymerization**

T. Hamada,[1] H. Igarashi,[2] T. J. Itoh,[3] T. Shimmen,[1] S. Sonobe[1]; [1]Department of Life Science, University of Hyogo, Hyogo, Japan, [2]Plant Science Center, Riken, Yokohama, Japan, [3]Division of Biological Sciences, Nagoya University, Nagoya, Japan

In plant cell, microtubules (MTs) organize various structures, such as cortical MT, preprophase band, spindle and phragmoplast. Microtubule-associated proteins (MAPs) play important roles in constructing and regulating these MT structures. Therefore the analysis of MAPs is needed in order to understand the function and regulation of MTs in plant cells. We succeeded in purifying a 200 kDa MAP (MAP200) from tobacco BY-2 cells using miniprotoplasts (evacuolated protoplasts), and have analyzed their function in tubulin polymerization. Analysis of the partial amino acid sequence showed that MAP200 was identical to the XMAP215/MOR1 family. Turbidity measurements of tubulin solution suggested that MAP200 promoted tubulin polymerization, and analysis by dark-field microscopy revealed that MAP200 increased both the number and length of MTs, suggesting that MAP200 has an ability to promote MT elongation and nucleation. To elucidate the mechanism of promotion of tubulin polymerization by MAP200, we performed turbidity measurement in the presence of GDP. It is known that the extent of tubulin polymerization is reduced depending on the concentration of GDP. In fact small amount of GDP in the assay sample significantly reduced the extent of tubulin polymerization. In the presence of MAP200, however, small amount of GDP have no apparent effect on the extent of tubulin polymerization. Both electron microscopy and experiments using a chemical crosslinker demonstrated that MAP200 forms a complex with several GTP-tubulin dimers. Surprisingly, the formation of complex between MAP200 and GDP-tubulin dimers was also found. We conclude that MAP200 could promote tubulin polymerization even in the presence of GDP. The analysis of MAP200-GDP-tubulin complex may provide a clue to understand the function of MAP200 in tubulin polymerization.

231

**Light Chain 3 (LC3) of Microtubule Associated Protein (MAP) 1A and 1B in Smooth Muscle Cell and Cardiomyocyte Differentiation**

N. S. Deshpande, G. M. Cann, M. Rabinovitch; Pediatrics, Stanford University School of Medicine, Stanford, CA

LC3, a marker of autophagy also binds fibronectin mRNA, increases the efficiency of its translation and as a consequence promotes smooth muscle cell (SMC) migration, necessary in the formation of a neointima in the developing ductus arteriosus and in diseased vessels. In the murine embryo,

expression of LC3mRNA is observed in limbs, lungs and brain, and is prominent at sites of neural crest cell differentiation, e.g., cardiac outflow, dorsal root ganglia and commissural neurons. We therefore hypothesized that LC3 might regulate differentiation of smooth muscle, cardiac and neuronal cells. Using embryonal carcinoma (P19) stem cells, we showed increased LC3 following differentiation to SMC but not cardiomyocytes. We also observed an increase in LC3 when neural crest (MONC-1) cells differentiated into SMC. However, overexpression of LC3 in P19 cells was not sufficient to induce SMC differentiation. To determine whether LC3 was necessary for differentiation, we stably transfected P19 cells with LC3 RNAi achieving a 70% knockdown, but differentiation to SMC, neuronal cells and cardiac myocytes was unimpeded. Despite reduced LC3 expression, differentiated cells exhibited characteristic markers, e.g., alpha SM actin, calponin, SM 22, SM-myosin heavy chain (MHC) in SMC;, desmin in cardiomyocytes; and, neurofilament and N-CAM in neuronal cells. Embryonic stem (ES) cells with deletion of both LC3 alleles also differentiated into SMC but not into cardiomyocytes in that no beating cells and no immunoreactivity for cardiac Troponin T, or slow MHC were seen. The absolute requirement for basal levels of LC3 in cardiomyocyte differentiation in embryoid bodies may be related to its requirement in translating a key regulatory protein.

232

**HDAC6 and Microtubules are Required for Autophagic Degradation of Aggregated Huntingtin**

A. Iwata,[1,2] B. E. Riley,[2] R. R. Kopito[2]; [1]Neurology, Graduate School of Medicine University of Tokyo, Tokyo, Japan, [2]Bio-X, Stanford University, Stanford, CA

CNS neurons are endowed with the ability to recover from cytotoxic insults associated with the accumulation of proteinaceous polyglutamine aggregates via a process that appears to involve capture and degradation of aggregates by autophagy. The ubiquitin-proteasome system protects cells against proteotoxicity by degrading soluble monomeric misfolded aggregation-prone proteins, but is ineffective against, and impaired by non-native protein oligomers. Here we show that autophagy is induced in response to impaired ubiquitin proteasome system activity. We show that ATG proteins, molecular determinants of autophagic vacuole formation, and lysosomes are recruited to pericentriolar cytoplasmic inclusion bodies by a process requiring an intact microtubule cytoskeleton and the cytoplasmic deacetylase HDAC6. These data suggest that HDAC6- dependent retrograde transport on microtubules is used by cells to increase the efficiency and selectivity of autophagic degradation.

233

**Functional Characterization of DdLIS1 and DdDCX, two Microtubule-associated Proteins involved in Lissencephaly**

I. Schulz, J. Faix, T. Ho, M. Rehberg, R. Gräf; A.B.I. Cell Biology, University of Munich, München, Germany

Lissencephaly results from impaired cell migration of neuronal precursors during development of the neocortex. Mutations in the genes encoding LIS1 and doublecortin (DCX) are responsible for approximately 80% of all cases of lissencephaly. Since *Dictyostelium* is an outstanding model for studies on cell migration and development, we analyzed the homologues of LIS1 and doublecortin. *Dictyostelium* LIS1 (DdLIS1) is a microtubule (MT) and centrosome-associated protein. Co-precipitation experiments revealed that DdLIS1 interacts with dynein, DdCP224 and the small GTPase Rac1A. Replacement of the DdLIS1 gene by the hypomorphic D327H allele had no strong effects on development but disrupted various dynein-associated functions and altered actin dynamics. Our results show that DdLIS1 is required for maintenance of the MT cytoskeleton, Golgi apparatus and nucleus/centrosome association, and they suggest that LIS1-dependent alterations of actin dynamics could also contribute to defects in neuronal migration in lissencephaly patients. DCX is characterized by two conserved tandem repeats of the DC-domain that are involved in microtubule (MT) binding. Although DCX has only been described in vertebrates where its expression was restricted to migrating, developing neurons, we have found a homologue in Dictyostelium. Its sequence similarity is mainly restricted to the DC-domains. GFP-DdDCX was localized along MTs and at the cell cortex. Endogenous DdDCX was distributed similarly but it was only detectable after 8 h of development. DdDCX null mutants exhibited no phenotype. However, DdDCX null mutants additionally carrying the hypomorphic DdLIS1 allele are defective in formation of streams and aggregates during development, although they show normal chemotaxis along external cAMP signals. Stream formation turned out to be independent of MTs. Taken together, this suggests that DdDCX and DdLIS1 cooperate in cAMP signaling and that both proteins may also be involved in cytoskeleton-independent functions in brain development.

234

**PDZ Domain of HtrA1 is Required for Intracellular Localization of HtrA1**

J. R. Chien,[1] R. Shridhar,[2] J. Staub,[1] V. Shridhar[1]; [1]Experimental Pathology, Mayo Clinic College of Medicine, Rochester, MN, [2]Radiation Oncology, Wayne State University School of Medicine, Detroit, MI

A candidate tumor suppressor HtrA1 is a PDZ containing serine protease and is a member of trypsin-like protease family of proteins initially described in bacteria to be essential for survival at elevated temperature; hence named High temperature requirement A (HtrA). Human HtrA1 was originally described as a secreted protein although cytoplasmic staining of HtrA1 was also reported. To clarify the subcellular localization of HtrA1, we performed subcellular fractionation and immunofluorescence staining. Interestingly, HtrA1 is detected both in membrane and cytosolic fractions. The membrane fractions contains 50 kD full-length HtrA1 whereas cytosolic fractions contain a unique 35 kD isoform as well as the 50 kD full-length HtrA1. Immunofluorescence staining with affinity-purified antibodies against HtrA1 confirms the cytoplasmic staining of HtrA1 co-localizing with microtubules. Localization of deletion mutant HtrA1 constructs indicates that PDZ domain is required for cytosolic localization. Whereas GFP-tagged full-length HtrA1 localizes to both endoplasmic reticulum and microtubules, GFP-tagged PDZ-deleted HtrA1 localizes exclusively to endoplasmic reticulum. In addition, co-culture of HtrA1 expressing cells with HtrA1-null cells led to cytosolic localization of HtrA1 in the null cell lines. Transient expression of HtrA1 in the null cell lines produces clusters of HtrA1-positive cells indicating the capacity of HtrA1 to transduce into neighboring cells. These evidences suggest that HtrA1 may contain the protein transduction domain. Consistent with this observation, HtrA1 contains a region rich in lysine and arginine in PDZ domain. These results point to the role of PDZ domain in protein transduction and the intracellular localization of HtrA1.

235

**Ase1p Stabilizes the Number and Distribution of Antiparallel Microtubules at the Spindle Midzone**

I. Loiodice, J. Czupryna, P. T. Tran; Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

Fission yeast ase1p belongs to the conserved ASE1/PRC1/MAP65 family of microtubule bundling proteins, which function to organize antiparallel midzone microtubules during mitosis, thus lending structural integrity to the bipolar spindle. Mitosis of fission yeast occurs in three phases: I) spindle formation, where the spindle is initially organized and reached length of ~3 um; II) metaphase and anaphase A, where the spindle remains at ~ 3 um long; and III) anaphase B, where the spindle quickly elongates to the final length of ~ 14 um. Live cell imaging showed that ase1p-GFP is recruited to the spindle during phase I, and then localized to the midzone during phase II and III. FRAP showed that ase1p-GFP at the spindle midzone is tightly bound to the microtubules, with the recovery half-time of t1/2 ~ 400 s. Quantitative fluorescence analysis of the mitotic spindles of wild-type cells showed that the spindle microtubules are symmetrically organized by the two spindle pole bodies (SPBs), with each opposite SPB nucleating similar number of microtubules during mitosis. In contrast, in ase1Δ cells the spindle microtubules appeared not symmetrical. There are often no clear midzones of microtubule overlapping regions in the ase1Δ cells. In addition, one SPB often nucleate more microtubules than its sister SPB. Without properly organized spindles and spindle midzones, ase1Δ cells exhibited a delay in phase II, and often showed premature spindle breakage during phase III. We propose that ase1p, by stabilizing antiparallel microtubules at the spindle midzone in a symmetrical manner, is in a pathway which regulate the number of SPB-nucleated microtubules.

236

### Binding Sites of Tau Repeat Motifs on Microtubules

A. Mitra,[1] D. Sept[2]; [1]Chemical Engineering, Washington University, St. Louis, MO, [2]Biomedical Engineering and Center for Computational Biology, Washington University, St. Louis, MO

Microtubule associated proteins, like tau, influence dynamic instability by stabilizing microtubules. Various isoforms of tau include a three to four 18-residue microtubule binding repeat separated by 13-14 residue inter-repeat. Experimental evidence for the structure of microtubule-bound tau has been unequivocal. With preassembled, taxol-stabilized microtubules decorated with tau, cryo-EM shows evidence for longitudinal binding of tau along individual protofilaments (Al-Bassam et. al, 2002), while atomic force microscopy at low tau to tubulin molar ratio indicates that tau oligomers form ring structures that encircle microtubules (Makrides et. al, 2003). In contrast, cryo-EM studies of microtubules copolymerized with tau in absence of taxol, suggest that tau and taxol binding sites overlap (Kar et. al, 2003). Employing stopped-flow kinetics and equilibrium competition binding experiments, two separate sites for tau binding has been proposed, namely, reversible binding of tau as seen with taxol-stabilized, preassembled microtubules, and secondly, nonexchangeable binding of tau under conditions of copolymerization of tau and tubulin (Makrides et. al, 2004). Recently, using metal shadowing and cryo-EM, tau has been identified to bind solely to the outside of microtubules both laterally and longitudinally (Santarella, 2004). None of these studies address the atomistic details of the interactions between the tau repeats and microtubules. In this study, using molecular dynamic simulations and molecular docking, we identified that the SKIGSTENLKHQ fragment of repeat motif R1 binds to beta-tubulin at the intra-dimer interface, as does the corresponding 12-residue fragment of repeat R4, consistent with ClustalW sequence alignment that R1 has higher homology to motif R1 than to other repeats. The corresponding fragments of repeat motifs R2 and R3 bind to alpha-tubulin near helix H12 and C-terminus of beta-tubulin. Our model supports longitudinal and lateral binding of tau to microtubules.

237

### Molecular Mechanisms of the Interaction between Tubulin and the Proteins of the Stathmin Family

I. Jourdain, M. J. Clement, S. Lachkar, B. Gigant, M. Knossow, A. Sobel, P. A. Curmi; IMBS, Palmerston North, New Zealand

Stathmin is a small cytosolic phosphoprotein which regulates microtubule dynamics by sequestering the microtubule subunit protein tubulin, thus preventing its incorporation into microtubules. The so-formed T2S complex is made of two ab tubulin heterodimers per stathmin molecule. Stathmin is the generic element of a protein family which includes RB3 that shares a highly conserved stathmin-like domain (SLD). Like stathmin, all SLDs are able to limit tubulin polymerization via the formation of T2S complexes, although the various complexes display different kinetics. The present work is aimed at understanding the mechanisms that lead to the SLD-specific interaction with tubulin. We dissected the role of three regions of the SLD by the means of recombinant fragments and chimeras of stathmin and RB3SLD. Physical biochemistry techniques allowed us to highlight that tubulin interacts co-operatively with stathmin. We also determined that the N-terminus and second tubulin-binding site regions make each SLD unique regarding tubulin binding. In parallel, X-ray crystallography and NMR data showed that the N-terminal region of SLDs forms a b-hairpin that caps the a-tubulin subunit in a region that mediates longitudinal tubulin-tubulin contacts. Together, this work has lead to the design of peptides from SLDs capable of mimicking the effect of the full-length SLDs on microtubule polymerization in vitro. Such peptides may contribute to the development of molecules that could slow down the cell cycle and be useful for the treatment of cancer.

## Cell Motility I (238-265)

238

### Actin Assembly, Contraction, and Adhesion during Spontaneous Polarization of Crawling Cells: Which of the Three Comes First?

S. Bohnet, S. Schaub, J. Meister, A. B. Verkhovsky; Lab of Cell Biophysics, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Cell polarization and directional locomotion are believed to involve asymmetry in actin assembly, contractility, and substrate adhesion. These asymmetries could develop in response to extracellular directional stimuli or as a result of spontaneous break of symmetry. We investigated random polarization in a model system of fish epidermal keratocytes. The cells were rendered isotropic and then induced to polarize by respectively depleting and restoring bivalent cations in culture media. We analyzed the distribution and dynamics of actin and myosin II using fluorescence speckling, conventional fluorescence microscopy and computer tracking, and studied the dynamics of substrate adhesions with interference reflection microscopy. The cells initially exhibited uniform actin assembly and retrograde flow of actin network in the lamellipodia all around the cell perimeter. Upon polarization, retrograde flow accelerated at the prospective rear of the cell, but actin assembly persisted at the rear at levels comparable with the assembly at the front long after the cell started to migrate directionally. In contrast to the smooth lamellipodia at the cell front, the lamellipodia at the rear developed filopodia-like ridges, which persisted as retraction fibers when the rear lamellipodia eventually retracted. We did not detect major differences in the density of myosin II at the prospective front and rear of the cell, but observed local areas of detachment from the substrate at the prospective rear. The results suggest that initial polarization results from asymmetry in actin retrograde flow, which is likely caused by detachment from the substrate in limited areas of the cell (focal detachments). Supported by Swiss National Science

Foundation grant 31-61589.

239

**Filament Packing Dynamics Generate Protrusive Force in Amoeboid Cell Motility**

L. Miao,[1] O. Vanderlinde,[1] J. Liu,[2] R. Grant,[3] A. Wouterse,[4] A. Philipse,[4] M. Stewart,[3] T. M. Roberts[1]; [1]Department of Biological Science, Florida State University, Tallahassee, FL, [2]Institute of Molecular Biophysics, Florida State University, Tallahassee, FL, [3]MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, [4]Van't Hoff Laboratory for Physical and Colloid Chemistry, Debye Institute, University of Utrecht, Utrecht, The Netherlands

Localized cytoskeletal assembly powers the leading edge protrusion in amoeboid cells but the mechanism by which this process pushes the membrane remains controversial. Amoeboid sperm of the nematode *Ascaris suum* extend a lamellipod that produces locomotion like that seen in conventional crawling cells, but the sperm movement is based on MSP filaments instead of actin. The MSP motility apparatus has been reconstituted in vitro; vesicles derived from plasma membrane trigger the assembly of MSP fibers to push the vesicle forward as they elongate. Every growing fiber exhibited a crescent-shaped region just behind the vesicle where the optical density was greater than elsewhere in the fiber. This dark crescent moved forward in concert with the vesicle as the fiber elongated and was located at the site where the force for vesicle movement is generated. We used electron tomography to show that the position of the dark crescent corresponded to the region where the filaments were more tightly packed. The packing of MSP filaments, which are relatively straight and have a persistence length of 9 µm, resembles that seen with rigid rods. The packing density of rods is inversely proportional to their aspect ratio, so longer rods pack less densely than shorter rods, suggesting that as filaments elongate their packing density decreases thereby generating a gel expansion that produces protrusive force. Consistent with this hypothesis, an engineered MSP mutant that generates shorter filaments results in higher filament packing density and slower movement. We propose that filament packing dynamics contribute generally to protrusive force generation in amoeboid cell motility. Supported by NIH grant GM29994.

240

**Active Bleb Formation Is Abated in *Lytichinus variagatus* Red Spherule Coelomocytes Upon Disruption of Acto-Myosin Contractility**

A. K. Sturycz, L. Winslow; Biology, Northwestern College, St. Paul, MN

Red spherule coelomocytes are one of five known immune cells in the sea urchin *Lytichinus variagatus*. These cells have a complex physiology and morphology, and have been previously characterized as motile $O_2$ transport cells (L.D. Winslow et al., MBC 14:128, 2003). Real time and time-laspse video microscopy of red spherule cells reveal a unique and dymanic array of cellular shapes and movements. Cells were observed to continuously send out large membrane extensions or blebs followed by extensive cytoplasmic streaming of cellular organelles and red pigmented granules which contain the the bacteriocide, echinochrome and the oxygen-binding protein hemoglobin. These cellular projections are not limited to movement along a substrate but occur in three dimensions. Western blot studies indicate that there is an abundance of cellular actin, myosin and tubulin within the cells. We have further examined the complex shape and motility by using a series of cytoskeletal disruption drugs. Treatment with 30µM cytochalasin B revealed a rapid and reversible change in both shape and motility. Cells appeared rounded having either a single spherical cell body with elongated membrane extensions or multiple spheres attached by short membrane extensions. Blebbing, intracellular organelle and granule motility appeared to cease. This attenuation of motility and blebbing was restored by washing out cytochalasin B. Treatment with phalloidin revealed a similar attenuation of motility. Treatment with 100µM blebbistatin showed complete and irreversible retraction of blebs and attenuation of all intracellular motility. Disruption of the microtubules using either nocodazole or taxol, showed no effect on cellular shape and motility. These results indicate that the acto-mysoin contractile mechanism is responsible for the dynamics of cell shape and membrane blebbing in invertebrate immune cells.

241

**Resveratrol Signals to the Actin Cytoskeleton to Promote Global Filopodia Extension and Inhibit Cell Migration**

N. G. Azios,[1,2] M. Harris,[1] L. Krishnamoorthy,[1] L. A. Cubano,[3] S. F. Dharmawardhane[1,2]; [1]Molecular Cell and Developmental Biology, University of Texas at Austin, Austin, TX, [2]Anatomy and Cell Biology, Universidad Central del Caribe, Bayamon, PR, [3]Anatomy and Cell Biology, Universidad Central del Caribe, Bayamon, PR

Resveratrol, a phytoestrogen in red wine, binds estrogen receptors α and β to exert both estrogenic and antiestrogenic effects. Our previous results show that while estrogen (E2) extends lamellipodia and promotes cell migration, resveratrol acts opposite by inducing filopodia and inhibiting cell migration. We hypothesize that the inhibitory action of resveratrol on cell migration may depend on its induction of sustained and global filopodia. The objective of this study is to delineate the mode of resveratrol signaling to the actin cytoskeleton. Therefore, we evaluated the role of Rho GTPases Rac and Cdc42 on resveratrol-induced filopodia extension. MDA-MB-231 cells stably expressing dominant negative Rac1(T17N) or Cdc42(T17N) were created and the effect of resveratrol on the actin cytoskeleton was analyzed. The ability of resveratrol to activate Rac1 or Cdc42 was monitored by pulling-down the active Rac1 or Cdc42 using a GST-PBD fusion protein. Stable expression of Rac1(T17N) did not affect resveratrol-induced filopodia extension, though resveratrol (5 µM) did induce Rac activity at 5-30 minutes. E2 did not induce lamellipodia in cells expressing Rac1(T17N) indicating that E2 signals via Rac to promote cell migration. Resveratrol (5 µM) was partially effective in filopodia extension in cells stably expressing Cdc42(T17N), but did not affect Cdc42 activity at 5-30 minutes. These data indicate novel Rac1- and Cdc42-dependent and independent signaling pathways for resveratrol-mediated modulation of the actin cytoskeleton leading to persistent filopodia extension and decreased cell migration. Since cell migration is a prerequisite for metastasis, resveratrol may act as a novel chemopreventive for metastatic breast cancer.

242

**A Role for ß4 Integrin in Directional Migration**

C. E. Pullar,[1] M. P. Marinkovich,[2] R. R. Isseroff[1]; [1]Dermatology, University of California, Davis School of Medicine, Davis, CA, [2]Program in Epithelial Biology, Stanford University School of Medicine, Stanford, CA

Wounding immediately generates an electric field (EF) that could be the earliest signal keratinocytes sense to initiate directional migration and re-

epithelialization. Indeed, keratinocytes migrate directionally towards the cathode (wound center) in an applied EF, a process termed galvanotaxis. While we have established a role for the epidermal growth factor receptor (EGFR) in galvanotaxis, the role of integrins is unknown. As a6ß4 integrin plays a role in keratinocyte chemotaxis and its ligand, laminin 5, is present at wound edges, does it play a role in keratinocyte galvanotaxis?. Immortalized ß4 negative keratinocytes were retrovirally transduced with a vector control (ß4-) or the ß4 gene (ß4+). Cells were starved of growth factors overnight and experiments were performed in the presence or absence of EGF (2ng/ml). Loss of ß4 has no effect on cell motility, while EGF increases it by 40%. However, ß4- cells are blinded to the EF and migrate randomly, while addition of EGF or re-expression of ß4 partially restores directional migration. This suggests a possible synergistic co-operation between EGF and ß4 integrin-mediated signals to initiate galvanotaxis. Loss of a 6ß4 ligation by laminin 5, either by transducing an adhesion-defective ß4 gene or addition of an anti- a 6ß4 antibody (ASC-8), increases motility in the presence of EGF while blinding the cells to the EF. Once more, the addition of EGF partially restores directional migration. Galvanotaxis requires formation of a new leading edge facing the cathode. Leading edge protrusion and adhesion is regulated by rac in many systems. Indeed, transduction of a constitutively active rac mutant into the adhesion-defective ß4+ cells fully restores galvanotaxis in the presence of EGF. It appears that keratinocyte EF-mediated directional migration, galvanotaxis, requires an EGF-mediated signal together with the ß4 integrin-mediated activation of rac.

243

**Characterization of Arf6 and FIP3 Interactions and their Role in Regulating Actin Dynamics at The Leading Edge of Migrating Cells**

E. Schonteich,[1] G. Simon,[1] H. T. Matern,[2] M. Pilli,[3] J. R. Junutula,[3] R. Prekeris[1]; [1]Cellular and Developmental Biology, UCHSC, Aurora, CO, [2]Exelixis, South San Francisco, CA, [3]Genentech, South San Francisco, CA

Emerging evidence has identified Arf6 as a key regulator of cytoskeletal remodeling and targeting membrane traffic during the transition of epithelial cells from polarized to a highly motile phenotype. While the role of Arf6 in regulating cytoskeleton is well established, little is known about the mechanism of Arf6 regulation during cell motility. Recently we have identified FIP3 as an Arf6 binding protein that may be involved in regulating Arf6 function (Fielding et al, 2005). What makes this discovery especially exciting is that FIP3 is also a Rab11-binding protein. Interestingly, Rab11 is known for its function in regulating endocytic membrane recycling to plasma membrane. Thus, it is tempting to speculate that FIP3 may play an integrative role between Arf6 and Rab11 by coupling Arf6-dependent cytoskeleton remodeling to Rab11-dependent targeted membrane transport to the leading edge of migrating cell. In this study we further characterize FIP3 and Arf6 interaction. We map FIP3-binding domain within Arf6 and use surface plasmon resonance to measure the affinities of FIP3 and Arf6 as well as FIP3 and Rab11 binding. Furthermore, we show that calcium binding to EF-hands regulates FIP3 conformation in vitro and its interaction with Arf6 in vivo. Finally, we show that FIP3 mediates the motility of MDA-MB-231 breast cancer cells by regulating Arf6 activation and actin stability at the leading edge.

244

**EphB/ephrin-B Interactions are Involved in Attachment and Spreading of Adult Human Dental Pulp Stem Cells**

A. Stokowski,[1,2,3] S. Gronthos, [3] S. Shi,[4] S. A. Koblar[2,1]; [1]School of Molecular and Biomedical Science, University of Adelaide, Adelaide, Australia, [2]ARC Special Research Centre for the Molecular Genetics of Development, Adelaide, Australia, [3]Division of Haematology, Institute of Medical and Veterinary Science, Adelaide, Australia, [4]Craniofacial & Skeletal Diseases Branch, National Institute of Dental & Craniofacial Research, National Institute of Health, Bethesda, MD

The inner chamber of the tooth, the dental pulp, is comprised of soft connective tissue derived from migrating neural crest cells during development. Dental pulp stem cells (DPSC) were isolated from the pulp of human adult third molar teeth. DPSC are capable of giving rise to pulp fibroblasts, odontoblasts, perivascular cells, adipocytes and preliminary evidence also suggests neural-like cells. The Eph family of receptor tyrosine kinases and their ligands, the ephrin molecules, play an essential role in the migration of neural crest cells during development. The present study examined the expression pattern and function of EphB/ephrin-B molecules on DPSC. Multiple receptors and ligands were identified on DPSC by real time PCR and immunohistochemistry. The function of EphB/ephrin-B molecules during DPSC attachment and spreading was assessed in the presence of Eph/ephrin-Fc fusion proteins using an established *in vitro* cell-spreading assay. In response to either EphB2-Fc or ephrin-B1-Fc, DPSC formed rounder and smaller cell bodies demonstrated by F-actin distribution, and restricted their ability to spread as indicated by time-lapse imaging. Additionally, immature focal adhesions were identified at the edge of the cell membrane, reminiscent of spreading initiation centers (SIC), which have only been observed at early stages of cell spreading. Inhibitor assay analysis indicated Eph forward signaling through the mitogen activated protein kinase (MAPK) pathway restricted DPSC spreading, while reverse signaling through the ephrin ligand was mediated via the phosphorylation of Src family tyrosine kinases, activating downstream signaling cascade. Collectively, these studies indicated that EphB/ephrin-B molecules provide an inhibitory environment for DPSC attachment, consequently resulting in the lack of spreading and detachment. These results may have implications for dental pulp development and regeneration.

245

**Ena/VASP Proteins Modulate the Shape of the Lamellipodial Leading Edge in Fish Keratocytes**

C. I. Lacayo, J. A. Theriot; Biochemistry, Stanford University, Stanford, CA

Ena/VASP proteins have been shown to play critical roles in actin dynamics and cell migration in several cell types including fibroblasts and *Dictyostelium discoideum*, where they influence cell speed and persistence. We are studying Ena/VASP proteins in the shape and migration of *Hypsophrys nicaraguensis* (Nicaraguan cichlid) epithelial keratocytes, which are among the fastest locomoting eukaryotic cells. Using polyclonal antibodies and GFP-tagged constructs, we have localized Ena/VASP proteins to focal adhesions in keratocytes found in epithelial sheets and to the leading edge of lamellipodia in polarized migratory keratocytes. Interestingly, VASP only appears to strongly localize to the “smooth” and not “rough” leading edge of lamellipodia. We have also found that keratocytes with strong localization of VASP at the “smooth” edge of lamellipodia have a distinct F-actin distribution compared to those keratocytes with “rough” lamellipodial edges. Additionally, treatment of cells with low levels of cytochalasin D delocalized VASP from lamellipodial edges and changed the F-actin distribution in the lamellipodia to more closely resemble the “rough” edge phenotype. We are currently examining how lamellipodial edge shape is affected by the delocalization of Ena/VASP proteins by competition with capping protein. We also examined the effect of mislocalizing Ena/VASP proteins in the motile behavior of keratocytes by using an Ena/VASP-sequestering construct targeted to mitochondria. We found that the mean speed, speed persistence, and trajectories of fish

keratocytes expressing the Ena/VASP-sequestering construct are not different compared to keratocytes expressing control constructs. Our results show that Ena/VASP proteins do not play an important role in determining the motile behavior of migrating keratocytes. However, Ena/VASP proteins are associated with the actin remodeling machinery that establishes the shape of the lamellipodial leading edge in crawling cells.

246

**Correlated Molecular Motions of Actin Cytoskeleton and Focal Adhesion Molecules within Adhesion Complexes**

K. Hu, L. Ji, G. Danuser, C. Waterman-Storer; Dept. of Cell Biology, Scripps Research Institute, La Jolla, CA

During cell migration, forces generated within the actin cytoskeleton by myosin contraction and actin polymerization at the cell front induce actin retrograde flow within the leading lamella. To investigate how the kinematics of the actin cytoskeleton are transduced to the ECM through Focal Adhesion complexes (FA), we imaged and correlated the kinematics of actin cytoskeleton with GFP-conjugated focal adhesion molecules in living cell using quantitative multispectral Total Internal Reflection Fluorescent Speckle Microscopy (TIR-FSM). The GFP- FA molecules fall in three categories based on the levels of organization within the FA, 1) actin binding proteins (alpha-actinin, talin, vinculin), 2) ECM binding proteins (αV/β3 integrins), and 3) "FA core" proteins, which do not directly interact with either actin cytoskeleton or ECM ( paxillin, zyxin and Focal adhesion kinase). To quantitatively describe the coupling between FA protein and the actin cytoskeleton, we first generate flow fields of actin and GFP-FA speckles by cross-correlating molecular motion in time, then calculate direction and speed correlation of the flow fields. Our results clearly show that FA components do not act concertedly as one unit, each displaying distinct kinematic behaviors. In particular, the actin binding FA components alpha-actinin, vinculin clearly have speckle motions that are highly correlated to actin motion indicating kinematic coupling between these molecules within FA. Other FA molecules examined display significantly lower level of coupling. Upon over-expression of constitutively active RacQ61L, we detect different degrees of increase in the coupling between actin and actin-binding FA molecules as well as FA core molecules, with the most pronounced effect on vinculin and talin motion within FA. FA actin binding proteins thus provide important regulatable linkages between actin and ECM.

247

**Kinetic Analysis of the Coupling between Temporal Production of Secondary Messengers and Pseudopod Projection in Cells Activated with Chemoattractant**

D. V. Zhelev, L. Vankova; Mechanical Engineering and Materials Science, Duke University, Durham, NC

Chemoattractants activate G protein coupled receptors (GPCRs) and induce cytoskeleton remodeling and chemotaxis. We have sown that the rate of pseudopod extension is dependent on ligand concentration (Chodniewicz and Zhelev, 2003, Blood 101:1181). We have also shown that the rate of pseudopod extension is dependent on the activity of kinases involved in the early stages of activation (Chodniewicz, Alteraifi and Zhelev, 2004, J. Bol. Chem. 279: 24460). This suggests that GPCRs are continuously managing the cytoskeleton remodeling. Here we explore the coupling between the production of secondary messengers and pseudopod projection. The temporal production of the secondary messenger PtdIns(3,4,5)$P_3$ is monitored by measuring the membrane translocation of PH-Akt-GFP or by measuring the phosphorylation of Akt/PKB in HL-60 cells. A kinetic model for PtdIns(3,4,5)$P_3$ production and Akt/PKB phosphorylation is developed in order to establish a temporal scale for comparison of the observed events. The model predicts that the apparent rate of production of secondary messengers during initial activation should be proportional to the apparent rate of increase of receptor occupancy or to ligand concentration. This is tested by measuring the rate of initial Akt/PKB phosphorylation in cells activated with either fMLP or SDF-1. Neutrophils express two receptors for fMLP: FPR1 is a high affinity receptor and FPR2 is a low affinity receptor, while SDF-1 is recognized by only one receptor CXCR4. The experimental data confirm that initial Akt/PKB phosphorylation is dependent on receptor occupancy and were capable of distinguishing between the dual receptor and single receptor activations. The dependence of the rate of initial pseudopod extension on ligand concentration was the same as the same dependence fpr Akt/PKB phosphorylation, however pseudopod extension was delayed by 20 to 30 s.

248

**Periodic Remodeling of the Actin Cortex Occurs in Two Mechanically Distinct Modes During Amoeboid Movement**

T. Soldati,[1] K. Yoshida[2]; [1]Biochemistry Department, University of Geneva, Geneva, Switzerland, [2]Department of Biological Sciences, Imperial College London, London, United Kingdom

It is widely believed that cell motility is caused by actin polymerisation directly pushing against the membrane at the leading edge producing filopodia and lamellipodia. Alternatively, cortical F-actin can counteract the cytoplasmic pressure generated by the contractile activity of myosin II at the posterior end. The latter has been revealed by studies of blebs, spherical hyaline protrusions that occur in stress or pathological situations when membrane detaches from its supporting cortex. The contribution of blebs to physiological cell motility has not been well appreciated. Using highg-resolution imaging, we found that Dictyostelium cells moving in a physiological but hypo-osmotic milieu are continuously blebbing. We quantitated cell motility and found that pseudopod extension, cell body retraction, and centroid movement were all decreased by an increase of osmolarity of the milieu in wild-type cells but not in myosin-II null cells. These observations suggested two mechanically different protrusive processes, characterised by blebs and filopodia, respectively. The bleb-mode becomes dominant when cells move at high speed under hypo-osmotic conditions, whereas the filopodium-mode becomes dominant when cells move at moderate speed under hyper-osmotic conditions, or when myosin II is defective. Spectral analysis, however, showed that cell movement has peaks at a fundamental frequency of 0.2 Hz and its harmonics, and that the power spectra of movement of blebbing cells are indistinguishable from those of non-blebbing filopodia-rich cells. In addition, cells in blebbing-mode chemotaxed as efficiently as cells in filopodium-mode, suggesting that the direction of blebbing is under the control of cAMP signalling in a way similar to filopodia extension. We therefore conclude that both bleb- and filopodia-modes occur through remodeling of the actin cortex driven by the same machinery but make use of different forces, fluid pressure and actin polymerisation, respectively.

249

**A WAVE2/Abi1 Complex Mediates CSF-1-induced F-actin Rich Membrane Protrusions and Migration in Macrophages**

W. G. Abou Kheir, J. Gevrey, H. Yamaguchi, B. M. Isaac, D. Cox; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Colony-stimulating factor 1 (CSF-1) is an important physiological chemoattractant for macrophages. The mechanisms by which CSF-1 elicits the formation of filamentous actin (F-actin) rich membrane protrusions and induces macrophage migration are not fully understood. In particular, very little is known regarding the contribution of the different members of the Wiskott-Aldrich Syndrome protein (WASP) family of actin regulators in response to CSF-1. Although a role for WASP itself in macrophage chemotaxis has been previously identified, no data was available regarding the function of WASP family verprolin-homologous (WAVE) proteins in this cell type. We found that WAVE2 was the predominant isoform to be expressed in primary macrophages and in cells derived from the murine monocyte/macrophage RAW264.7 cell line (RAW/LR5). CSF-1 treatment of macrophages resulted in WAVE2 accumulation in F-actin rich protrusions induced by CSF-1. Inhibition of WAVE2 function by expressing a dominant-negative mutant in RAW/LR5 cells or introducing anti-WAVE2 antibodies in primary macrophages and RAW/LR5 cells as well as reduction of endogenous WAVE2 expression by RNA-mediated interference (RNAi) in RAW/LR5 cells resulted in a significant reduction of CSF-1-elicited F-actin protrusions. WAVE2 was found in a protein complex together with Abelson kinase interactor 1 (Abi1) in resting or stimulated cells. Both WAVE2 and Abi1 were recruited to and necessary for the formation of F-actin protrusions in response to CSF-1. Reducing the levels of WAVE2, directly or by targeting Abi1, resulted in an impaired cell migration to CSF-1. Altogether these data identify a WAVE2/Abi1 complex crucial for the normal actin cytoskeleton reorganization and migration of macrophages in response to CSF-1.

250

**Dynamic Relationships Among PI 3-kinase Signaling, Cell Spreading, and Asymmetric Membrane Protrusion Following the Attachment of Fibroblasts to Surfaces**

M. Weiger, J. Haugh; Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC

Cell adhesion and migration are essential steps in numerous biological processes such as wound healing. Cell spreading and migration involve both surface attachment and cytoskeletal reorganization, leading to membrane extension and ultimately cell polarization, processes which are stimulated and coordinated through intracellular signal transduction pathways mediated by adhesion and chemoattractant receptors. In fibroblasts and many other cell contexts, activation of the phosphoinositide (PI) 3-kinase pathway is strictly required for motility. PI 3-kinases generate specific 3' PI lipid products, which act as membrane second messengers, and the spatial pattern of 3' PI density in the membrane is thought to control the directionality of membrane protrusion and cell migration. As a model system, we have quantitatively followed the time course of PI 3-kinase activation, membrane spreading and post-spreading motility of mouse fibroblasts following their initial attachment to fibronectin and polylysine coated surfaces. Two imaging modes, total internal reflection fluorescence and epifluorescence microscopies, were used in conjunction with fluorescent 3' PI and nonspecific probes to evaluate the relationships among these processes for various plating conditions. We report the following findings: 1) spontaneous PI 3-kinase activation generally foretells the dramatic acceleration of cell spreading rate at localized regions of the contact area; 2) cells plated on fibronectin versus poly-lysine show similar qualitative behavior in this respect but spread at different rates; and 3) after the initial spreading achieves a steady state, cells often exhibit transient oscillations in PI 3-kinase activity that coincide with localized membrane protrusions, which may play an important role in establishing polarity for migration.

251

**WASP-Interacting Protein (WIPa) is Required for Filopodia Formation and Prompt Chemotactic Response to a New Gradient**

S. A. Myers, J. W. Han, L. Leeper, S. Gruver, C. Y. Chung; Pharmacology, Vanderbilt Univ. Med. Ctr., Nashville, TN

WASP family proteins activate F-actin assembly via stimulation of the Arp2/3 complex and have been shown to be necessary in diverse cellular processes including chemotaxis. In this study, we examined the role of WASP-interacting protein (WIP) in the process of WASP-mediated actin assembly during chemotaxis of *Dictyostelium*. Mutations of the WH1 domain of *Dictyostelium* WASP led to a reduction in binding to a newly identified *Dictyostelium* homolog of mammalian WIP, WIPa, reduced F-actin localization at the leading edge of chemotaxing cells, and led to a reduction in chemotactic efficiency. WIPa translocates to cortical membrane upon uniform cAMP stimulation in a PI3 kinase-dependent manner and to sites of newly forming filopodia as well as the leading edge of chemotaxing cells and WIPa appears to colocalize with WASP at the leading edge of chemotaxing cells, presumably resulting in the stimulation of F-actin assembly as well as filopodia formation. WIPa increases F-actin nucleation and elongation both *in vivo* and *in vitro* in a WASP-dependent manner. WIPa overexpressing cells exhibited higher levels of leading edge F-actin content, defects in chemotactic efficiency, and more filopodia. Reduced expression of WIPa by expressing a hairpin-WIPa (hp-WIPa) construct resulted in hyperpolarized cells and these cells exhibit a small, but significant increase in mean velocity and chemotactic index, indicating that these cells are moving more straightforward to the cAMP source than wild type cells. However, hp-WIPa cells exhibit a delayed response to the new chemoattractant source due to delayed formation of filopodia toward the new gradient. These results suggest that WIPa and WASP allow cells to reorient toward a new gradient promptly by initiating localized bursts of actin polymerization and/or elongation.

252

**Tuba: A Cdc42 GEF that drives NWASP-dependent Actin Assembly**

E. M. Kovacs, F. B. Gertler; Biology, Massachusetts Institute of Technology, Cambridge, MA

Rapid rearrangement of the actin cytoskeleton underlies normal cellular motility. Tuba is a multidomain scaffolding protein that functions to bring together cellular trafficking and signaling pathways with actin regulatory proteins. Tuba has an internal BAR domain, is a GTPase exchange factor (GEF) for Cdc42, and binds directly to NWASP through a C-terminal SH3 domain. Overexpression of Tuba exacerbates dorsal ruffling observed in B16 melanoma cells. Ruffles form and dissipate rapidly and continuously with an apparent random distribution over the entire surface of the cell. Tuba puncta are observed to localize to the base of these short-lived dorsal ruffles coincident with NWASP, Cdc42, Arp2/3 and actin. Treatment of cells with wiskostatin, a specific inhibitor of NWASP, results in dissolution of Tuba puncta and a cessation of ruffling. Additionally, ruffling requires functional BAR, GEF and C-terminal SH3 domains of Tuba, and a dynamic actin cytoskeleton. Ruffling shows no obvious association with endocytosis using TR-Dextran as a fluid phase marker and has no apparent colocalisation with caveolin. Motile cells undergo morphological changes that result in the formation of distinct plasma membrane protrusions such as dorsal ruffles and invadopodia. Tuba is a key component of the molecular machinery driving the formation of these processes.

253

**Tonic Protein Kinase A Activity Maintains Inactive Beta 2 Integrins in Unstimulated Neutrophils by Reducing Myosin Light Chain Phosphorylation: Role of Myosin Light Chain Kinase and Rho Kinase**

C. D. Chilcoat, Y. Sharief, S. L. Jones; Clinical Sciences, North Carolina State University, Raleigh, NC

Activation of beta 2 integrins is necessary for neutrophil adhesion and full activation of neutrophil effector functions. We demonstrated previously that inhibition of PKA activity in quiescent neutrophils is sufficient to increase beta 2 integrin cell surface expression, affinity, and adhesion. Thus, a tonic level of PKA activity present in unstimulated neutrophils prevents inappropriate activation of beta 2 integrins. Myosin light chain (MLC) phosphorylation is an important regulator of leukocyte integrin function and adhesion. Moreover, PKA regulates MLC phosphorylation via inhibiting myosin light chain kinase (MLCK), and MLC dephosphorylation via effects on the Rho kinase/myosin light chain phosphatase pathway. We hypothesize that the tonic inhibitory effect of PKA upon beta 2 integrin activation neutrophils operates via its inhibition MLC phosphorylation. We demonstrate here that that inhibition of PKA activity with KT5720 activated beta 2 integrins and adhesion coincident with an increase in myosin light chain Ser 19 phosphorylation. KT5720-induced activation of beta 2 integrins, adhesion, and MLC Ser 19 phosphorylation was abolished by pretreatment with MLCK inhibitor ML-7, a specific MLCK inhibitory peptide, and the Rho kinase inhibitor Y-27632. These findings demonstrate that tonic PKA activity prevents activation of beta 2 integrins and adhesion by dampening MLC phosphorylation via a mechanism that operates by inhibiting both MLCK and Rho kinase signaling pathways.

254

**Mechanics of Neutrophil Phagocytosis**

M. Herant,[1] V. Heinrich,[2] M. Dembo[1]; [1]Biomedical Engineering, Boston University, Boston, MA, [2]Biomedical Engineering, University of California, Davis, CA

A complete picture of phagocytosis requires an understanding of the mechanical events that lead to cellular deformation, protrusion of a phagocytic cup, and eventual formation of a phagosome. We study the mechanics of FcgammaR-driven phagocytosis of antibody-coated polystyrene beads (diameters from 3 um to 11 um) by initially spherical neutrophils, and we present side by side microscopic observations of real neutrophils and results from finite element computer simulations of virtual neutrophils with realistic cytoskeletal, cytosolic and membrane dynamics. These comparisons are used to assess the viability of various mechanistic models for neutrophil phagocytosis with special attention to the time evolution of the cell morphology, of the motion of the bead, and of the cortical tension. We find that the optimal models involve two key mechanical interactions: a repulsion between cytoskeleton and free membrane that drives protrusion, and an attraction between cytoskeleton and membrane newly adherent to the bead that flattens the cell into a thin lamella with retrograde centripetal cytoskeletal flow. Other models based on phenomena such as cytoskeletal expansion or swelling appear to be ruled out as main drivers of phagocytosis because of the characteristics of bead motion during engulfment. The transitions in cell shape observed to take place during phagocytosis can be successfully interpreted via the interplay of interior cytoplasmic viscosity and surface cortical tension. We finally show that the protrusive force necessary for the engulfment of large beads points toward a large scale (0.5 um) strain energy stored in the cytoskeleton, and that the flattening force can plausibly be generated by the known concentrations of unconventional myosins at the leading edge.

255

**F-Actin Levels in Furrow Canals, but not Basal Junction Integrity, is Critical for Drosophila Cellularization**

A. Sokac,[1] E. Wieschaus[1,2]; [1]Department of Molecular Biology, Princeton University, Princeton, NJ, [2]Howard Hughes Medical Institute, Princeton, NJ

Drosophila cellularization is a modified form of cytokinesis, during which the plasma membrane invaginates around each of 6000 cortical nuclei to generate an epithelial sheet of adherent cells that exhibit apical/basal polarity. The process thus requires specialized proteins to coordinate simultaneous polarity establishment, cell-cell junction assembly and F-actin remodeling with a large-scale membrane invagination event. Nullo is a basic (pI 11.4), myristoylated protein that is essential for the initiation and progression of cellularization. Nullo localizes to the basal cell-cell junction that assembles during early cellularization; and in *nullo* loss-of-function embryos (*nullo*X) both basal junctions and the adjacent F-actin/Myosin-2 furrow canals (FC) are disrupted. Since it was previously suggested that basal junctions are required for FC formation and/or stability, we examined embryos deficient for the basal junction component β-catenin. These embryos do not assemble basal junctions. Nonetheless, cellularization proceeds with normal FCs that are functional as indicated by their Myosin-2-based constriction in late cellularization. Quantitative analysis of confocal images further revealed that FCs in β-catenin-deficient embryos contain wild-type levels of F-actin throughout cellularization. We, thus conclude that basal junction disruption cannot account for the FC phenotype in *nullo*X embryos and instead Nullo may have a direct influence on F-actin remodeling at forming FCs. Indeed, F-actin levels in *nullo*X versus wild-type FCs is significantly decreased throughout cellularization. The FC disruption in *nullo*X mutants likely results from this decrease in F-actin levels as the phenotype is mimicked by treating wild-type embryos with low doses of cytochalasin-D. Furthermore, over-expression of Nullo increases F-actin levels in FCs. Thus Nullo modulates F-actin levels in forming FCs; and the F-actin level in FCs, but not basal junction integrity, is critical for cellularization to initiate and proceed.

256

**A Deeper Look into *Dictyostelium* PAKa's Posterior Cortical Localization**

S. Zhang, R. A. Firtel; Section of Cell and Developmental Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA

In *Dictyostelium*, PAKa is an important effector in the signaling pathways required for proper chemotaxis. PAKa and myosin II co-localize to the posterior cortex of chemotaxing cells, which suggests that PAKa might indirectly regulate myosin II assembly and contractility. This was confirmed by analysis of PAKa's null phenotype. The N-terminus of PAKa was previously shown to be essential for PAKa's posterior localization. To explore the mechanism that regulates PAKa's localization, we created a series of truncated PAKa constructs fused to GFP and examined their subcellular localization in chemotaxing cells. We showed that the first 244 residues of the PAKa N-terminus are necessary and sufficient for PAKa's posterior cortical localization. Deletion of residues 1-40 caused a diffuse, cytosolic distribution of truncated PAKa(s) with no enrichment at the cell cortex. In contrast, the removal of amino acids 207-244 resulted in a loss of the posterior localization with the reporter localizing

uniformly along the cortex in unstimulated cells and enriched in the leading edge of chemotaxing cells. This finding suggested that the domains containing residues 1-40 and 207-244 might directly or indirectly associate with F-actin and myosin II filaments, respectively, leading to PAKa's localization in the posterior cortex. To further identify potential adaptor proteins linking PAKa to actomyosin, we performed a yeast two-hybrid screen and identified several proteins that interacted with the N-terminus of PAKa, including: *Dictyostelium* CBP3, an actin-associated calcium binding protein; sactin, a previously unidentified protein with actin-like and filamin-like domains; and ZAK1, an upstream regulator of *Dictyostelium* GSK3. Currently, we are analyzing these potential interacting proteins and their possible roles in controlling polarized PAKa distribution. We would like to thank Alan Kimmel for ZAK1 constructs and strains and Hyung-Soon Yim & San-Ouk Kang for CBP3 constructs and strains.

257

### Polarized Dynamics of Single Molecule G-protein Coupled Receptors in *Dictyostelium* Chemotaxis

S. de Keijzer,[1] A. Serge,[1] P. H. M. Lommerse,[1] F. van Hemert,[1] G. Lamers,[2] H. Spaink,[2] T. Schmidt,[1] B. Snaar-Jagalska[2]; [1]LION, Leiden University, Leiden, The Netherlands, [2]IBL, Leiden University, Leiden, The Netherlands

The directed cell movement, chemotaxis, of cells in response to shallow gradients of chemoattractants involves gradient sensing, polarity and motility. Recent evidence suggests that dynamic organization of proteins and lipids plays an important role. Based on confocal imaging, the signalling components of the chemotaxis pathway (like PI3K, PTEN, AC, CRAC, Akt/PKB, PhdA) in *Dictyostelium discoideum* have a polarized distribution upon cAMP stimulation. However, the cAMP receptor, cAR1, and the coupled G-protein β subunits are uniformly distributed along the cell surface. Since localization is not the only dynamic property of a receptor, the behaviour of single cAR1 on the apical membrane was followed in real time with Single Molecule Microscopy, where normal wide-field microscopy with laser excitation is combined with ultra-sensitive CCD camera detection. This gives us the possibility of detecting signals with a positional accuracy of 40 nm. We measured the diffusion of cAR1-YFP under different physiological conditions. In resting cells there were two diffusion fractions, a fast fraction with D = 0.19 $\mu m^2/s$ (38%) and an immobile fraction (62%). In polarized cells exposed to cAMP gradient we found a difference in the diffusion-behaviour between the anterior and the posterior. At the anterior a larger number of mobile receptors were observed. This motility shift was not because the fluidity of the membrane changed nor because of conformational changes of the receptor due to phosphorylation. We show that the motility shift of the receptors at the leading edge resembles the uncoupling/activation of Gα2-protein. We suggest a model in which the polarized dynamics of cAR1 amplifies the signal at the leading edge and accelerates intracellular signalling. This model adds a new mechanism explaining establishment of polarization upon gradient sensing during *Dictystelium* chemotaxis.

258

### A Centrin-based Cellular Spring that Generates nNs of Force

D. C. France,[1] V. Baru,[2] M. Shribak,[3] S. Inoue,[3] S. McCutcheon,[4] H. E. Buhse,[4] P. T. Matsudaira[1,2,5]; [1]Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, [2]Whitehead Institute for Biomedical Research, Cambridge, MA, [3]Marine Biological Laboratory, Woods Hole, MA, [4]Biological Sciences, University of Illinois at Chicago, Chicago, IL, [5]Biology, Massachusetts Institute of Technology, Cambridge, MA

The spasmoneme of the unicellular protozooan *Vorticella convallaria* is among the fastest and most powerful cellular engines known. A contractile organelle that uses calcium instead of ATP for energy, the spasmoneme is a bundle of fibers of undetermined composition. Centrin is the major component by dry-weight and the active calcium-binding element. Also found in filamentous structures associated with various actions ranging from spindle pole and centrosome movements to contraction of whole cell bodies, centrin may be the basis of a fourth cytoskeleton. To understand how centrin-based systems are involved in cell motility, we have been characterizing the structure, mechanics, and biochemistry of the spasmoneme and report several novel findings. First, the spasmoneme is a powerful cellular engine. We imaged spontaneous contractions while imposing a centrifugal force load on the cell body. At accelerations over 10000g, the cell body becomes tear-drop shaped, its contents become stratified, and yet the stalk still contracts. We estimate an upper bound on the amount of force generated during contraction in excess of 200 nN. Second, the current rubber-band model of the spasmoneme relies on a previously observed loss of spasmoneme birefringence in the contracted state, as oriented, birefringent fibers become disoriented with added calcium. In contrast, images from the orientation-independent polarized light microscope (LCPolScope) show the spasmoneme consists of fibers that retain some degree of order in the contracted state. Finally, we are able to correlate the structure and mechanics of the spasmoneme directly with centrin. After extracting the stalks under conditions which retain reversible calcium-dependent contractility, we find that one anti-*Vorticella* centrin antibody (anti-centrin 5) abolishes contractility while another (anti-centrin 4) does not. These observations suggest that the spasmoneme is a centrin-based mechanism that is unlike any actin or microtubule-based cellular engine, yet more powerful.

259

### Microtubule Targeting to Filopodia

J. M. Schober, Y. Komarova, O. Chaga, G. G. Borisy; Cell and Molecular Biology, Northwestern University, Chicago, IL

Microtubules are essential for formation of cell polarity and directed cell movement. Through alternating phases of shortening and growth, microtubule ends actively probe target sites in protrusive lamellipodia. Microtubules interact with filopodia in neuronal growth cones (A. Schaefer et al. 2002. *J. Cell Biol.* 158:139-152) and target adhesion sites in fibroblasts causing adhesion site dissociation (I. Kaverina et al. 1999. *J. Cell Biol.* 146:1033-1043). In our studies we identified interaction between microtubules and filopodia in B16F1 melanoma cells and NIH 3T3 fibroblasts. Following adhesion to fibronectin, cells exhibited protrusive lamellipodia with numerous filopodia. B16F1 cells that showed entry of microtubules into lamellipodia were scored for positive targeting events, defined as intersection of a microtubule end with the base of a filopodium. Analysis of digital images revealed targeting events at much greater frequencies than expected by chance. Live cell imaging demonstrated interaction between microtubules and filopodia in cells transfected with yellow fluorescent protein linked to tubulin and fascin, a specific marker of filopodia. Time-lapse sequences show multiple targeting events to filopodia at the protrusive edge of cells and microtubules buckling after targeting. Total internal reflection fluorescence microscopy images showed microtubules in close proximity to and below filopodia. Focal adhesion kinase and phosphotyrosine were not detected at microtubule target sites, suggesting interaction between microtubule ends and filopodia is independent of focal adhesions. To evaluate microtubule growth, cells were transfected with GFP-CLIP170, a plus-end binding protein that

highlights actively polymerizing microtubules. The GFP-CLIP170 reporter showed that microtubules actively polymerize along filopodia. These studies provide evidence for cross-talk between microtubules and filopodia, and suggest a potential mechanism by which microtubules may play an active role in cell protrusion. Supported by Training Grant no. NIH 2T32AG260-06 (JS) and NIH GM25062 (GGB).

260

**Focal Adhesions Area is Affected by Microtubule Acetylation**

A. Tran,[1] S. Che,[1] T. Yao,[2] J. Bulinski[1]; [1]Biological Sciences, Columbia University, New York, NY, [2]Pharmacology and Cancer Biology, Duke University, Durham, NC

Microtubules are subject to several reversible post-translational modifications, including acetylation. While the function of microtubule acetylation is unknown, manipulating the activity of HDAC6, a microtubule-selective deacetylase, has been shown to affect polarized cell migration. Overexpression of HDAC6 increases cell motility [1], while the specific inhibition of HDAC6 decreases cell motility [2]. We corroborated and expanded these results, observing that transformed cells are more potently affected than non-transformed ones. The role of microtubules in cell movement is still under investigation; however one possible way that microtubules might influence motility is through regulating focal adhesions [3]. Therefore, we hypothesized that changes in microtubule acetylation via modulation of HDAC6 activity would result in changes in focal adhesions. We monitored focal adhesions by quantifying paxillin localized to adhesions and found that short-time treatments of Trichostatin A (TSA), a general HDAC inhibitor, resulted in a time-dependent increase in focal adhesion area. Furthermore, washout of TSA resulted in a reversal, focal adhesion area returned to control levels. In cells over-expressing HDAC6, focal adhesion area was significantly decreased, compared to control cells, while in cells where HDAC6 had been knocked down by siRNA, focal adhesion area was significantly increased. Thus, manipulation of microtubule acetylation through changes in HDAC6 activity quantitatively alters the cell's focal adhesions, although whether the mechanism involves altered elaboration or turnover of adhesions is thus far unknown. This change in focal adhesion, perhaps or in cooperation with other mechanisms, is a likely contributor to the observed change in cell motility upon modulation of HDAC6. [1]

261

**Regulation of the MSP Polymerization that Generates Leading Edge Protrusion in *Ascaris* Sperm**

K. Yi,[1] S. M. Buttery,[1] M. Stewart,[2] T. M. Roberts[1]; [1]Department of Biological Science, Florida State University, Tallahassee, FL, [2]MRC laboratory of Molecular Biology, Cambridge, United Kingdom

Leading edge protrusion in the amoeboid sperm of *Ascaris suum* is driven by localized assembly of the MSP cytoskeleton. This process is orchestrated by an integral membrane phosphoprotein, MPOP, but also requires additional cytosolic components. MSP-based protrusion has been reconstituted *in vitro*; vesicles derived from the sperm plasma membrane are pushed by elongation of a columnar meshwork of MSP filaments, called a fiber. The motility apparatus contains a MW ~34 kDa protein (p34) that has a conserved kinase domain. This protein co-localized with MPOP at the vesicle-fiber interface, the site of MSP polymerization and protrusive force generation in the *in vitro* motility system. Moreover, binding assays showed that p34 interacts specifically with phosphorylated MPOP and also with a 47 kDa cytosolic protein that we designated MSP fiber protein 3 (MFP3). Immunolabelling demonstrated that MFP3 is localized throughout the fiber, and functional assays demonstrated that MFP3 is necessary for maintaining fiber structure. Sequence analysis suggests that MFP3 is a serine/threonine phosphoprotein. These results indicate that MPOP, p34 kinase and MFP3 may form a complex to nucleate and regulate MSP polymerization at the membrane surface. Supported by NIH Grant GM 29994.

262

**Characterization of the Roles for the Na,K-ATPase Alpha and NHE Isoforms in Mouse Sperm Motility**

S. Chakraborty, A. Sengupta, P. F. James; Zoology, Miami University, Oxford, OH

The ion signaling pathways that activate and maintain sperm motility are multifactorial and remain incompletely resolved. Recent studies using inhibitors and ionophores have demonstrated a role for the rat Na,K-ATPase alpha4 isoform, in conjunction with one or more NHE isoforms, in maintaining sperm intracellular pH and motility. Three NHE isoforms (NHE1, NHE5, and spNHE) and two Na,K-ATPase alpha isoforms (alpha1 and alpha4) are expressed in rodent sperm. Targeted deletion of spNHE results in immotile sperm and infertility in mice however deletion of NHE1 doesn't affect fertility. No information concerning the role of NHE5 in sperm motility is available. The existence of a functional mouse Na,K-ATPase alpha4 isoform necessary for maintaining sperm motility, as in the rat, has not been demonstrated and the role that the Na,K-ATPase alpha1 isoform plays in sperm motility is unknown. Furthermore, the functional relationships between the Na,K-ATPase and NHE isoforms found in sperm are unresolved. The availability of Na,K-ATPase alpha1 heterozygous and homozygous NHE1 and spNHE knockout mice provides a unique set of reagents to dissect out the individual roles played by the Na,K-ATPase alpha and NHE isoforms in sperm. Here, we have cloned the cDNA and characterized the mouse Na,K-ATPase alpha4 isoform protein. As in rat, this mouse protein is expressed in the sperm flagellum and is necessary for normal motility. We have also determined that loss of all NHE1 or 50% of Na,K-ATPase alpha1 does not alter motility suggesting that these isoforms play roles unrelated to motility or that other isoforms compensate for their loss. Interestingly, we have demonstrated an upregulation of the Na,K-ATPase alpha4 protein, but not alpha1 or NHE5, in NHE1 knockout testes suggesting a functional relationship for NHE1 and the Na,K-ATPase alpha4 isoform in sperm physiology.

263

**Localization and Developmental Expression of the Human Na,K-ATPase Alpha4 Isoform in Sperm**

A. Sengupta,[1] S. Chakraborty,[1] T. Hlivko,[2] P. F. James[1]; [1]Zoology, Miami University, Oxford, OH, [2]Pathology, Suma Health System/ACH, Akron, OH

The Na,K-ATPase is an integral transmembrane protein, minimally composed of an alpha and a beta subunit. In humans three alpha isoforms (α1, α2, and α3) have been well documented. In rodents a fourth isoform (α4) has been identified and shown to be critical to maintaining sperm motility likely via its effects on a $Na^+/H^+$ exchanger (NHE) found in sperm. Recently we have characterized the human Na,K-ATPase α4 isoform and localized its expression to sperm in human testes. Here we have demonstrated the expression of the alpha4 protein in human sperm lysates but not in human kidney, skeletal muscle and brain lysates by Western blot analysis and specifically localize the Na,K-ATPase alpha4 protein to the

principle piece of the human sperm flagellum using immunofluorescence. In addition, we demonstrate that the Na,K-ATPase α4 expression appears only after sexual maturity in humans: the alpha4 protein is present in the sperm flagellum in testes from a 16 year old individual but not in testes from 3 month, 6 year, 9 year, or 10 year olds. This data demonstrates that the expression of the human alpha4 protein is developmentally regulated and its expression occurs with the appearance of mature sperm. Specific inhibition of the rat Na,K-ATPase alpha4 isoform with the cardiac glycoside ouabain results in loss of sperm motility. Motility can be restored to alpha4-inhibited sperm with ionophores that move $H^+$ suggesting that this isoform is essential to the activity of a NHE which regulates intracellular sperm pH. Here we demonstrate restoration of motility to alpha4-inhibited sperm using ammonium chloride or the cAMP analogue 8-Br-cAMP. This data further supports the link between Na,K-ATPase alpha4 inhibition-induced loss of sperm motility and alteration in intracellular pH and suggests that this occurs via a cAMP-mediated pathway.

264

**Spatial Localization of M-Calpain to the Plasma Membrane by $PIP_2$ binding is Required for EGF Receptor-Mediated Activation**

H. Shao,[1] J. Chou,[1] D. Stolz,[2] C. J. Baty,[2] A. Wells[1]; [1]Pathology, University of Pittsburgh, Pittsburgh, PA, [2]Cell Biology, University of Pittsburgh, Pittsburgh, PA

Cell motility is critical for wound repair and tumor invasion. Calpain activity is required for rear de-adhesion during both haptokinesis and chemokinesis of fibroblasts and epithelial cells. Growth factors activate m-calpain (CAPN2) via the ERK MAP (mitogen-activated protein) kinases, but only when these kinases are localized to the plasma membrane. We thus hypothesized that m-calpain is activated by epidermal growth factor (EGF) only when it is juxtaposed to the plasma membrane secondary to specific docking site(s). Osmotic disruption of NR6 fibroblasts demonstrated a significant portion of m-calpain being complexed with the substratum-adherent membrane; the amount of m-calpain at the membrane increased in an EGF-dependent manner. In vitro, purified m-calpain bound to nylon-immobilized phosphoinositides but not other phospholipids. This was mirrored in vivo as removal of phosphoinositide bisphosphate ($PIP_2$) by bacterial phospholipase C released the bound m-calpain. This binding capacity was found to reside in isolation to Domain III of calpain, which has a putative C2-like domain. Activation of m-calpain by phosphorylation at serine 50 was found to increase m-calpain binding to the membrane. In vivo, downregulation of phosphoinositide production by 1-butanol, resulting in diminished PIP2 in the plasma membrane, and eliminated EGF-induced calpain activation but not MEK activity. Immunostaining and immunoblotting of the membrane footprints showed that PI-PLC and 1-butanol limited the membrane association of m-calpain. Importantly, we found m-calpain was accumulated at the rear of both intact cell and plasma membrane footprint in wound healing area; EGF enhanced this m-calpain accumulation to the rear. These data support a model of m-calpain binding to $PIP_2$ to enable ERK activation, and provides a mechanism by which cell de-adhesion is directed to cell body and tail as PLCγ hydrolyses PIP2 in the protruding lamellipodia.

265

**Proliferation and Chemotaxis of *Dictyostelium* Amoebae are Inhibited by Naringenin, a Flavonoid Compound Enriched in Grapefruits**

M. Russ, R. Martinez, P. Steimle; Biology, University of North Carolina at Greensboro, Greensboro, NC

Naringenin, a flavanone compound, has been shown to affect critical cellular processes such as glucose uptake, cell division, and possibly cell migration; all of these processes are involved in the cancer cell phenotype. As a result, there has been considerable interest in defining the mechanisms by which naringenin exerts its effects on cells. In this study, we have used the social amoeba, *Dictyostelium discoideum*, as a model system for examining the cellular processes and signaling pathways affected by naringenin. We found that *Dictyostelium* cell division is inhibited in a dose-dependent manner by naringenin ($IC_{50}$~100 μM). Naringenin-based inhibition of *Dictyostelium* proliferation does not appear to be due to cell death since cell viability is not affected by growth-inhibiting levels of naringenin. Naringenin (50 μM) also inhibits aggregation of *Dictyostelium* cells during the early stages of multi-cellular development, suggesting that this compound may interfere with chemotactic signaling. Parallel under-agarose assays showed that naringenin inhibits *Dictyostelium* chemotaxis towards folic acid. Numerous studies in *Dictyostelium* have established that the localized synthesis of phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) by PI3 kinase is a crucial early step in the chemotaxis signaling pathway. Since naringenin is an effective inhibitor of PI3 kinase activity, we explored that hypothesis that naringenin inhibits *Dictyostelium* chemotaxis by blocking PI3 kinase activity. To this end, we assayed live *Dictyostelium* cells for chemoattractant-stimulated translocation of a $PIP_3$-specific PH domain in the presence and absence of naringenin. Surprisingly, we found naringenin did not alter the rapid and localized increase in $PIP_3$ synthesis in response to chemoattractant stimulation of *Dictyostelium* cells, suggesting that naringenin may affect cellular migration via a previously unrecognized mechanism.

## Centrosomes I (266-281)

266

**G1 Cell Cycle Progression After Centrosome Removal**

Y. Uetake,[1] C. N. English,[1] A. Khodjakov,[2] G. Sluder[1]; [1]UMass Medical School, Worcester, MA, [2]Wadsworth Ctr, Albany, NY

Several studies indicate that the centrosome is required for G1 cell cycle progression in untransformed mammalian somatic cells. However, the fact that Hela cells, finishing mitosis without a centrosome, progress through S into the next mitosis demonstrates that centrosomes are not absolutely required for G1 progression in transformed cells (La Terra et al. J Cell Biol. 168:713, 2005). Presumably suppression of p53 and/or other aspects of transformation abrogate the mechanisms that detect centrosome loss or damage. To further investigate the involvement of the centrosome in G1 cell cycle progression we microsurgically remove the centrosome from hTERT-RPE1 cells stably transfected with human centrin-1/GFP. These cells have normal p53 function, because DNA damage leads to a rise in p53 levels and cell cycle arrest. When we remove the centrosome as early as 1 hour into G1, all karyoplasts (acentrosomal cells) progress through interphase into mitosis and divide. Surprisingly, the daughter karyoplasts incorporate BrdU and continued to divide (25/26). We then cut cells in G2 to remove one centrosome. 20 cells enter mitosis within 6 hours after the cut, assemble a spindle lacking one centrosome, and divide in a normal bipolar fashion to give a daughter with a centrosome and one without a centrosome. Of the acentriolar daughters, 10 progress to another mitosis, 4 arrest in G1 and 6 arrest in G2. Additionally, the acentrosomal karyoplasts form 0-8 bright focal centrin spots after centrosome removal in G1 or G2, suggesting *de novo* centriole assembly. These results provide the first indication that normal p53 positive human cells may progress through G1 after centrosomal removal and form centrioles *de novo*.

267

**Cryo Em Tomography and Averaging of Assymetric Structures: Application to γTuRC and the Microtubule Minus End**

J. M. Lyle, P. Koenig, D. A. Agard; Biochemistry & Biophysics, UCSF, San Francisco, CA

Microtubules are nucleated from the centrosome by the gamma-tubulin ring complex (γTuRC), a 2.2 MDa protein complex with a lock washer shape complementing the end of the microtubule (MT). While this complex is found free in the cytosol, its nucleation activity is largely restricted to the centrosome. In order to gain insights into the mechanisms of nucleation by the γTuRC and the origins of the functional differences between solution and bound to the centrosome, I am working to determine the structure of the microtubule nucleation site as it actually exists in the centrosome and to compare it to the structure of the isolated γTuRC. Using cryo electron microscopic tomography it is possible to directly visualize the nucleation machinery at the MT minus ends at about 5nm resolution. In an effort to better define the nucleation site, I am helping to develop software designed to find, align and average the minus end density, with a goal of reaching 2.5nm resolution. Our current reconstructions clearly show the γ-TuRC cap and also reveal the fibrous nature of the pericentriolar material (PCM). Using isolated γTuRCs and similar reconstruction approaches, I am building a higher resolution structure of this template of microtubule nucleation. My current structure, at approximately 2.5nm resolution, demonstrates the lock washer shape of the γTuRC, likely made up from the lateral association of γ-tubulin small complexes (γTuSC) and the existence of an additional cap structure that may act to stabilize the ring of γTuSCs. Comparisons of the three-dimensional structures of γ-TuRC will be discussed along with their implications for the mechanism of nucleation.

268

**Kinesin-like Protein Binding to a Conserved Domain in Gamma-tubulin Regulates Spindle Assembly and Mitotic Exit**

J. L. Paluh,[1] A. S. Rodriguez,[1] K. Mohan,[1] C. L. Mayer,[1] A. N. Killilea[2]; [1]Biology, Boston College, Chestnut Hill, MA, [2]Lawrence Berkeley National Labs, Berkeley, CA

In eukaryotes, correct bipolar spindle assembly and fidelity in chromosome segregation requires the coordinated action of kinesin-like proteins and is monitored by Mad2p, a component of the spindle assembly checkpoint. At kinetochores, the kinesin-like protein CenpE plays important roles both in chromosome segregation and in the spindle assembly checkpoint. What role kinesin-like proteins play at microtubule ends of spindle poles, possibly through interactions with microtubule organizing center proteins, has not been determined. In addition, localization of Mad2p to poles preceding anaphase, is conserved but is not understood. Members of the ubiquitous Ncd/Kar3 family of kinesin-like proteins are implicated in regulation of microtubule number, organization, minus-end dynamics and γ-tubulin function. Our findings indicate that in fission yeast the kinesin-like protein Pkl1p binds to γ-tubulin to regulate both bipolar spindle assembly and post-metaphase localization of Mad2p to poles. Former models propose Ncd/Kar3 family kinesin-like proteins exert forces primarily along interdigitating microtubules, with this action deterring that of similarly positioned BimC motors in pushing spindle poles apart. We identified residues on γ-tubulin for Pkl1p binding. These represent a subset of invariant residues present in the same domain of β-tubulin but absent in α-tubulins. Mutation of this γ-tubulin domain to disrupt Pkl1p binding is sufficient to rescue bipolarity in a *cut7-22* (BimC) mutant and mimics *pkl1Δ*. Distal chromatin-microtubule interactions also contribute to oppose Pkl1p. We present a revised three-point model for bipolar spindle assembly based on genetic, molecular biochemical and cell biological analysis and discuss implications of our work on kinesin-like protein binding to γ-tubulin versus β-tubulin families. We propose that Pkl1p at poles, similar to CenpE at kinetochores, may play dual roles in regulating microtubule minus-ends and providing a link to Mad2p.

269

**Dynein and Dynactin are Involved in Anchoring of Microtubules at the Centrosome in Interphase Cells**

O. Kovalenko,[1,2] E. Nadezhdina,[2] V. I. Rodionov[1]; [1]Cell Biology, University of Connecticut Health Center, Farmington, CT, [2]Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russian Federation

Cytoplasmic dynein and its cofactor dynactin are known to be involved in the establishment and maintenance of the centrosomal radial microtubule (MT) array in interphase cells, but their roles in the attainment of the radial MT organization is not clear. It has been suggested that dynein/dynactin complex contributes to this process by transporting MT nucleation sites to the centrosome in the form of gamma-tubulin/pericentrin complexes and possibly by directly inducing MT nucleation at the centrosome. It has also been found that dynactin can participate in the anchoring of MT minus ends at the centrosome. To examine the role of dynein/dynactin in the MT radial array maintenance in interphase cells, we studied the behavior of a MT radial array in Vero cells labeled with Cy3-tubulin and injected with dynein/dynactin inhibitors, a function-blocking dynein antibody, or recombinant p50 subunit of dynactin that disrupts the dynactin complex. We found that both inhibitors induced rapid (~60 min) loss of radial MT organization in most of the injected cells without affecting the levels or localization pattern of major centrosomal proteins gamma-tubulin, pericentrin, and ninein, suggesting that dynein/dynactin are not involved in transport of these components to the centrosome after the radial array establishment. Moreover, MT nucleation rates at the centrosome measured by counting of the number of EB1-GFP comets emerging from the centrosmal region also remained unchanged in the presence of the inhibitors, indicating that MT nucleation on the centrosome does not require the intact dynein/dynactin complex. We conclude that while both dynein and dynactin are essential for the maintenance of the radial MT organization, their primary role is independent of MT nucleation and consists of anchoring of the minus ends of the MTs nucleated at the centrosome.

270

**Analysis of the Interaction Between *Drosophila* Pericentrin Like Protein (D-PLP) and the Dynein Complex**

N. Peel, T. M. P. Barros, E. Lucas, J. W. Raff; Wellcome CR UK Gurdon Institute, Cambridge, United Kingdom

The *Drosophila* pericentrin like protein (D-PLP) is localised to centrioles and pericentriolar material (PCM). In fixed *d-plp* mutant larval brains PCM components are not efficiently localised to the centrosome (Martinez-Campos et al, 2004). We have undertaken a live analysis of the basis for this defect, in embryos. We generated germline clone embryos that lack D-PLP and also express GFP-fusions of PCM components. We show that, in the absence of D-PLP, PCM components are still recruited to the centrosome, but cannot be retained there. Instead, they are dispersed away from the centrosome in a microtubule dependent process. Thus, D-PLP appears to maintain the structural integrity of the centrosome by counteracting microtubule dependent dispersal of the PCM. How does D-PLP fulfil this role? It has been shown previously that vertebrate pericentrin interacts

with the cytoplasmic dynein complex. We are testing whether this interaction is conserved in fly, and if so, if it has a functional significance in the maintenance of PCM integrity. To this end, we have developed a new GFP-dynein-complex marker that we will use, in addition to p50-GFP, to test whether D-PLP plays a role in dynein localisation.

271

### Interaction of Cep135 with Centrosomal Proteins

K. Kim, S. Lee, J. Chang, K. Rhee; Department of Biological Sciences, Seoul National University, Seoul, Republic of Korea

In interphase cells, the centrosome acts as a microtubule organizing center for cell shaping, polarity and motility. In mitotic cells, it plays as the spindle pole for segregation of chromosomes. The centrosome consists of a pair of centrioles and the pericentriolar material. With a long term goal to understand protein networks of the centrosome, we have investigated specific associations among centrosomal proteins, including Cep135. The results suggested that Cep135 might function as a scaffold for maintenance of the centrosome structure.

272

### Characterization of a Centrin-Binding Protein in the Human Centrosome

J. Azimzadeh,[1] P. Hergert,[2] U. Euteneur,[3] L. Daviet,[4] A. Khodjakov,[2] M. Bornens[1]; [1]Umr144/cnrs, Institut Curie, Paris, France, [2]New York State Department of Health, Wadsworth Center, Albany, NY, [3]Institute for Cell Biology, LMU-ABI, Munich, Germany, [4]Hybrigenics SA, Paris, France

Centrin proteins are small calcium-binding proteins conserved in a wide range of eukaryotes. They have been detected in many different cellular structures, suggesting that they could have several distinct functions. Many of these seem to derive from an ancient association of centrin proteins with the flagellar apparatus. In yeast and human, centrins have been involved in centrosome duplication, but the precise mechanisms in which they are implicated remains elusive. To better understand the functions of centrin proteins in the human centrosome, we have used a two-hybrid approach to identify centrin-interacting proteins. This has led to the identification of p65 protein. Like centrin, the p65 protein is an ancient protein conserved in various eukaryotic lineages. p65 protein homologues are only found in the genomes of species that assemble canonical centrioles, reflecting a possible function in centriole structure assembly. p65 protein interacts with both ubiquitously expressed human centrin proteins, Cen2p and Cen3p, as confirmed by immuno-precipitation experiments and GST-pull downs assays. A specific antibody reveals an asymmetric staining of the two centrioles and shows that the protein is recruited to the forming pro-centrioles during late S or G2 phase. Immunogold-EM shows that, like centrin, p65 is localized in the distal part of the centriole lumen. RNAi inactivation of p65 did not reduce the staining of the mother centriole, suggesting a stable incorporation in the centriole structure. p65 staining of the daughter centriole was however suppressed, and cell viability strongly reduced in HeLa cells, whereas in RPE1 cells, an arrest in G1 was observed. Electron microscopy shows that most depleted cells present short pro-centrioles, suggesting a possible role of p65 in the assembly or maturation of the distal part of centrioles.

273

### Identification and Characterisation of the Novel Centrosome Component BC1

S. Elliott, J. Sante, T. Stearns; Biological Sciences, Stanford University, Stanford, CA

The centrosome is the major microtubule organising centre of the cell. Centrosomes are central to cytoplasmic organisation, chromosome segregation and cell division. The centrosome consists of two centrioles surrounded by pericentriolar material. The sperm basal body is very similar to the centrosome of somatic cells, however the sperm basal body does not contain many of the pericentriolar components of the centrosome. In an attempt to identify and analyse core centrosome components we fractionated sperm and focused on the fraction containing centrin. Centrin is a highly conserved protein known to localize to centrioles. Using mass spectrometry, we found that other known centrosome components and a number of uncharacterized proteins co-fractionated with centrin. One of these proteins, BC1, was chosen for further analysis. BC1 is a coiled-coil protein conserved in higher eukaryotes. BC1 localises to the sperm basal body and the centrosome in tissue culture cells. Purification of TAP-tagged BC1 from tissue culture cells has identified interacting partners and RNAi is being conducted to explore the function of BC1 in mammalian cells.

274

### CenpJ is a Novel Protein Required for Centriole Assembly

R. Basto,[1] J. Lau,[1] N. Peel,[1] C. G. Woods,[2] J. W. Raff[1]; [1]Wellcome Trust Cancer Research Gurdon Institute, University of Cambridge, United Kingdom, [2]Department of Medical Genetics, CIMR, University of Cambridge, United Kingdom

Centrosomes are the major microtubule-organizing centers (MTOC) of animal cells and as such play a role in various microtubule-related functions, including cell shape, motility and division. A centrosome consists of a pair of centrioles surrounded by pericentriolar material (PCM). Centrioles not only have an important role in organizing the PCM, they also can act as basal bodies, specialised structures that localise at the base of all cilia and flagella. The growing list of centrosome-associated proteins suggests that centrosomes could be involved in previously unsuspected cellular functions and centrosome dysfunctions could therefore contribute to the development of human diseases. CenpJ is a novel centrosome protein and mutations in the *cenpJ* locus in humans is thought to contribute to microcephaly, a neurodevelopmental disorder where brain size is reduced. We are currently using the genetic organism, *Drosophila melanogaster*, to investigate the cellular function(s) of CenpJ. We have found that CenpJ is localised to the centrioles throughout the cell cycle. Antibody injection experiments revealed that CenpJ is essential for both centriole replication and PCM recruitment.

275

### Dissecting the Centrosome Positioning Pathway

J. L. Feldman, W. F. Marshall; Biochemistry, University of California, San Francisco, San Francisco, CA

Although originally named for its central location within the cell, the centrosome often resides in more peripheral locations. Relocation of the centrosome within the cell, for example, during wound healing or migration, requires the shuttling of the centrosome to specific locations. Furthermore, centrioles, the core of the centrosome, also need to be targeted to specific cellular sites. For example, centrioles are localized to the apical end of the cell in order to make a cilium or flagellum. These movements are especially important during development, where the precise placement of a cilium in various cell types is critical. In the unicellular green alga *Chlamydomonas reinhardtii*, centrioles must be shuttled from

their central position during mitosis to their apical location during interphase. In order to understand how a centrosome is specifically positioned within a cell, we have employed a novel screen in *Chlamydomonas.* This screen takes advantage of the fact that properly positioned centrioles are required in order for cells to phototax; cells with aberrantly placed centrioles, and therefore aberrantly placed flagella, do not have the defined geometry between the eyespot (the light sensing organelle of the cell) and the flagella that is required for phototaxis. We have screened 10,000 lines for phototactic defects and have uncovered a number of interesting mutants that have defects in centriolar assembly and number. In addition, we have also uncovered a class of mutants, termed askew (asq), in which the centrioles are mislocalized within the cell. In asq mutants, centrioles lose their characteristic apical localization within the cell and are instead shifted to more lateral locations. Characterization of these mutants has helped us gain insight into the molecular mechanism by which centrosomes are properly localized and has revealed the extent of the instructive role of the centrosome in determining cellular geometry.

276

**Proteomic Analysis of Isolated *Chlamydomonas* Centrioles and Further Characterization of Candidate Proteins**

L. C. Keller,[1] E. P. Romijn,[2] I. Zamora,[1] J. R. Yates,[2] W. F. Marshall[1]; [1]Biochemistry, UCSF, San Francisco, CA, [2]The Scripps Research Institute, La Jolla, CA

The centriole is one of the most enigmatic organelles in the cell. Centrioles are cylindrical, microtubule-based barrels found at the core of the centrosome. Centrioles also act as basal bodies during interphase to nucleate the assembly of cilia and flagella. Much of the mystery surrounding centrioles stems from the fact that until recently, the protein composition of centrioles was largely unknown. We exploited *Chlamydomonas* to conduct the first direct proteomic analysis of isolated centrioles. Through a bioinformatics cross-validation method we were able to group candidate centriole proteins into two groups: Basal body proteins with Upregulated Genes (BUGs), which are likely to be involved in ciliogenesis, and Proteome Of Centriole proteins (POCs). Our proteomic analysis provides the groundwork for further analysis of proteins involved in assembly and maturation of the centriole. To test if candidate proteins are bona fide centriole components, GFP-fusion proteins are made and localized in Hela cells. To elucidate a centriole assembly pathway, shRNA constructed against each candidate protein are transfected into Hela cells to examine centriole defects. Preliminary results suggest that candidate proteins are necessary for centrosome stability.

277

**Tandem Affinity Purification in *Dictyostelium*: Search for Interactors of EB1 and DdCP224 at the Centrosome and the Microtubule Plus-ends**

K. V. Koch,[1] Y. Reinders,[2] A. Sickmann,[2] R. Graef[1]; [1]Cell Biology, A. Butenandt Institute LMU, Muenchen, Germany, [2]Protein Mass Spectrometry and Functional Proteomics Group, Rudolf-Virchow-Center for Experimental Biomedicine, Würzburg, Germany

EB1 and DdCP224 are centrosomal components that also localize to microtubule (MT) tips and interact with each other. DdCP224 is involved in centrosome duplication and cytokinesis whereas EB1 assists in spindle formation. At the MT tip these two proteins are part of a complex that links MTs to the cell cortex. DdCP224 and EB1 were shown to interact with dynein. In addition, DdCP224 coprecipitates with dynactin and DdLIS1. To obtain more detailed information on the interactions of these proteins tandem affinity purification of cytosolic protein complexes containing DdCP224 and DdEB1 was conducted. EB1 was used in its full length. DdCP224 is split into two parts to be able to distinguish between centrosomal and cortical interactors, since the C-terminus is sufficient for centrosomal binding whereas a cortical localization can be detected with an N-terminal construct. Mass spectrometrical analysis of the purified complexes was used to identify novel interactors of these proteins. Additionally, this approach verified the known interaction of EB1 and DdCP224 as well as an interaction of DdCP224 with tubulin. An interaction between TACC and the C-terminal part of DdCP224 was detected and similar to findings in *Drosophila*, the TACC domain is sufficient for the centrosomal localisation of the protein and the interaction with DdCP224.

278

**Identification and Characterization of Novel Centrosome Components**

D. Ducat,[1,2] S. Kawaguchi,[2] Y. Zheng[1,2]; [1]Johns Hopkins University, Baltimore, MD, [2]Carnegie Institution of Washington/HHMI, Baltimore, MD

Recent research is revealing an increasing role for the centrosome in regulating various cellular activities, including formation of cilia, control of cell cycle and organization of the mitotic spindle. Despite this, the molecular nature of the centriole and the surrounding pericentriolar material (PCM) remains poorly understood. In order to gain a better understanding of the components required for the assembly and function of the centrosome, we have isolated and characterized a fraction of Drosophila embryo extract that is enriched in centrosome complementing activity. This fraction is capable of restoring microtubule nucleation capacity to centrosomes that have been stripped of many centrosome-associated proteins by high-salt treatments. Mass-spectroscopy analysis has revealed 360 proteins in this fraction, including a number of known centrosome proteins. We have undertaken a phenotype-based RNAi screen using Drosophila S2 cells in order to identify proteins in this fraction that are involved in the regulation of the centrosome. Preliminary analysis has revealed a number of candidate proteins that may have previously unrecognized roles in regulating centrosome-related functions.

279

**The Use of Vertebrate Reverse Genetics in the Study of the Centrosomal TACC Proteins**

A. Barr,[1] F. Langevin,[2] F. Gergely[3]; [1]Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom, [2]MRC Laboratory Of Molecular Biology, Cambridge, United Kingdom, [3]Department of Oncology, University of Cambridge, The Hutchison/MRC Research Centre, Cambridge, United Kingdom

The Transforming Acidic Coiled-coil Containing (TACC) family of proteins are centrosomal proteins characterised by a highly conserved TACC domain at their C-terminus, which is essential for their centrosomal localisation. There is only one TACC protein in flies while three TACC proteins have been identified in mammals and birds (TACC1, 2 and 3). Previous data from the laboratory revealed an interaction between the TACC proteins and the colonic and hepatic Tumour Overexpressed Gene (ch-Tog), a member of the XMAP215/dis1 microtubule-stabilising family. More recently, we described that TACC3 and ch-Tog co-operate in the formation of the bipolar spindle in mammalian cells. Our understanding however is still incomplete as for the functions of, and the relationship between, the vertebrate TACC proteins. To explore this

question, we are using the chicken B-cell line, DT40, that allows targeted gene disruption via homologous recombination. Our strategy is to knock out the TACC domain of each of the three chTACC proteins and hence create cell lines that carry one or more disrupted *tacc* genes; the ultimate aim being to obtain cells without any functional TACC protein. We found that knocking out TACC2 in chicken cells does not affect either the viability or the morphology of the cells, an observation consistent with published studies on the TACC2 knockout mouse. We are in the process of generating cell lines where TACC2 is mutated in combination with TACC1 or TACC3.

280

**HCA66 is a Novel Protein Associated with the Centrosome from S to M-phase**

X. Fant,[1] A. Merdes[2]; [1]Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom, [2]UMR2587, CNRS, Toulouse, France

Several proteins exhibit a dynamic association with the centrosome in a cell cycle dependent manner. For example, gamma-tubulin has been shown to accumulate temporarily at the centrosome before mitosis, resulting in an increase of microtubule nucleation during spindle formation. To investigate changes in the composition of the pericentriolar material during the cell cycle, centrosomes were isolated at different stages, such as G1 and S phase. Pericentriolar material was prepared by salt-stripping of centrosomes, and analyzed by gel electrophoresis followed by MALDI-TOF mass spectrometry. We identified HCA66, a novel protein that localizes to the centrosome as well as to the nucleolus. Antibodies were raised against recombinant HCA66. Consistent with biochemical data, immunofluorescence microscopy revealed that HCA66 is recruited to the centrosome in S phase and remains associated until the end of anaphase. The sequences responsible for centrosomal and nucleolar localization were mapped using GFP-fusion constructs. The first 86 amino-acids of HCA66 (GFP-HCA66-1-86) are sufficient to confer a centrosomal localization. Overexpression of GFP-HCA66-1-86 leads to an aggregation of the exogenous protein in the cytoplasm and to a loss of gamma-tubulin signal at the centrosome in most cells, impairing microtubule regrowth after cold induced depolymerization. Preliminary siRNA experiments show that decrease of HCA66 expression leads to cell death, suggesting an essential role of the protein.

281

**A Centrosome-Associated RNA Encoding RNA-Directed Nucleotide Polymerase**

M. C. Alliegro,[1] M. A. Alliegro,[1] R. E. Palazzo[2]; [1]Cell Biology, LSU Health Sciences Center, New Orleans, LA, [2]Biology, Rensselear Polytechnic Institute, Troy, NY

The centrosome in animal cells is composed of a pair of centrioles surrounded by a relatively ill-defined pericentriolar matrix. Centrosomes serve as the major microtubule organizing center (MTOC) in cells, carry the mother centriole which is the basal body progenitor, and organize the assembly of the mitotic spindle during cell division. In spite of their obvious significance and over 100 years of research, our understanding of centrosome composition, organization, and mechanisms of action is poor when compared with other organelles. Centrosomes duplicate only once per cell cyle to ensure development of a normal bipolar spindle. The initial event in centrosome duplication is centriole replication, which is generative, semiconservative, and independent of the nucleus. These and other observations have led to the proposal that centrosomes contain their own complement of nucleic acids, possibly representative of an organellar genome as has been described for mitochondria and chloroplasts. The consensus in the field is that centrosomes do not contain DNA, but may contain RNA. We have extracted a unique set of RNAs from isolated surf clam centrosomes (cRNAs) and show that the first transcript to be analyzed in depth contains a conserved RNA-directed nucleotide polymerase domain and is concentrated in, if not fully restricted to centrosomes. These results support the hypothesis that centrosomes contain an intrinsic complement of RNAs, and provide new opportunities to address this five decades-old question.

## Intracellular Movement (282-303)

282

**A Computational Model of Actin-dependent Melanosome Transport in Fish Retinal Pigment Epithelial (RPE) Cells**

D. Tacelosky,[1] S. Forman,[2] S. Cooper,[2] P. Heil,[2] C. King-Smith[1]; [1]Department of Biology, Saint Joseph's University, Philadelphia, PA, [2]Department of Math and Computer Science, Saint Joseph's University, Philadelphia, PA

Retinal pigment epithelial (RPE) cells from fish contain melanosomes, melanin-rich pigment granules, which undergo light-dependent migrations from the cell body through long apical projections in response to light. *In vitro*, exogenous cAMP triggers unidirectional melanosome aggregation, while cAMP washout with dopamine produces saltatory, bidirectional melanosome dispersion. Both aggregation and dispersion are dependent on the actin cytoskeleton, and isolated melanosomes support motility of actin filaments in *in vitro* motility assays, suggesting a role for myosin motors in melanosome movements. To better understand the mechanism of dispersion, we applied kinetic parameters of melanosome dispersion to a discrete-event computer simulation. Excursion time, length, and velocity data were collected from time-lapse microscopy videos and entered into the input analyzer of the Arena© discrete event simulation package. The lognormal distribution, which consistently generated a good fit to the data, was used in the simulation, which was written in C. The simulation treats all melanosomes as individual entities that begin fully aggregated at time 0, and progressively disperse in a series of randomized events. At the completion of an event, the simulation is updated with respect to melanosome position before advancing to the most imminent event. Simulation output closely mirrored the behavior of individual melanosomes during dispersion. For a three-dimensional rendering of melanosome dispersion, simulation algorithms were translated into programming codes for the computer visualization program, Alice©. The lognormal distribution used in the model describes the process of “multiplicative degradation”, where the amount of degradation accumulates, reaches a critical point, and results in failure. In the context of melanosome dispersion, “failure” might represent complete disengagement of myosin motors from the melanosome, or release of motors from the actin track, resulting in the observed saltatory motility.

283

**Identification of AKAPs Involved in Regulation of Pigment Transport in Melanophores**

I. V. Semenova, P. Ivanov, V. Rodionov; Cell Biology, University of Connecticut Health Center, Farmington, CT

Aggregation and dispersion of pigment granules in melanophores is regulated by cAMP through the Protein Kinase A (PKA) signaling pathway.

We have recently shown that regulatory subunit of PKA is associated with pigment granules, where it forms complexes with molecular motors involved in pigment transport. We have also shown that association of PKA regulatory subunit with pigment granules occurs via binding to two A-Kinase Anchoring Proteins (AKAPs) with molecular masses ~80 kDa and 160 kDa. Here we have selectively extracted the two AKAPs from pigment granules and developed an experimental strategy for theis isolation and identification. Using this strategy we were able to isolate the 80kDa AKAP and identify it by mass spectrometry. We are now identifying the high molecular weight AKAP. The structure and molecular interactions of these proteins will provide insights into the molecular mechanisms of protein scaffolding on pigment granules that is critical for regulation of intracellular organelle transport.

284

**Pigment Organelle Tranport in Zebrafish - Using Forward Genetics to Identify Regulators of Molecular Motor Proteins**

L. Sheets,[1] E. M. Mellgren,[2] J. A. Kuhlman,[3] J. S. Eisen,[3] S. L. Johnson,[2] B. J. Schnapp[1]; [1]Cell and Developmental Biology, Oregon Health & Science University, Portland, OR, [2]Genetics, Washington University School of Medicine, St Louis, MO, [3]Institute of Neuroscience, University of Oregon, Eugene, OR

Fish melanophores are a valuable model system for investigating the molecular mechanisms of organelle transport because they aggregate or disperse their pigment granules (melanosomes) rapidly and coordinately in response to changes in intracellular levels of cAMP. Dispersion of melanosomes to the cell periphery occurs when intracellular cAMP levels are elevated and protein kinase A (PKA) is activated. Conversely, aggregation of melanosomes to the cell center occurs when cAMP levels are lowered and a phosphatase is activated. The downstream targets of PKA and the phosphatase are still unknown. We are interested in identifying these targets because they are likely regulators of molecular motor protein function. Previous studies in melanophores have employed species for which a forward genetic approach is impractical. The objective of our study is to use a forward genetic screen in zebrafish (*Danio rerio*) to identify proteins that regulate melanosome transport downstream of known signalling molecules. We have developed a screening assay that specifically identifies zebrafish mutants with melanosome transport defects. One of the mutants we have identified, *nermal*, is unable to fully disperse its melanosomes. We have analyzed melanosome movement in isolated *nermal* melanophores and our results indicate that the defective gene acts downstream of PKA and is involved in regulating microtubule based tranport. A second identified mutant, b867, is unable to evenly disperse its melanosomes. Analysis of isolated b867 melanophores indicates that the gene is involved in the instigation of actin-based transport. Our future aim is to identify the genes responsible for defective melanosome movement and uncover the function of these genes in organelle transport regulation.

285

**Alpha-herpesvirus Tegument Protein UL37 is Required for Intracellular Capsid Transport in Axons**

J. I. Lee, G. A. Smith; Microbiology/Immunology, Northwestern University, Chicago, IL

Alpha-herpesvirus infections of the vertebrate nervous system result in recurrent peripheral diseases or, less commonly, fatal encephalitis. The microtubule-based transport of capsids in axons is a critical determinant of disease, but occurs by an unknown mechanism. Previous work in our laboratory has demonstrated that actively transported capsids are associated with two viral tegument proteins, VP1/2 and UL37. We have made a recombinant virus lacking UL37 and expressing a red-fluorescence protein tagged capsid. Motions of capsids were monitored in both Vero cells and primary dorsal root ganglion neurons. Compared to the parent virus, capsids of the UL37-null virus displayed a defect in capsid transport. Future studies will focus on the mechanism of UL37-based intracellular capsid transport.

286

**Herpesviruses Use a Vesicular Transport Mechanism to Reach the Distal Axon**

S. E. Antinone, G. A. Smith; Microbiology-Immunology, Northwestern University, Chicago, IL

Alpha-herpesvirus infection of the vertebrate nervous system requires distinct steps of anterograde and retrograde transport in axons. The mechanism of axonal transport has, in part, been obscured by contradictory findings regarding whether capsids egress in axons as naked or enveloped particles. Determining the site of mature viral particle formation within neurons is not only critical to the mechanism of microtubule transport but is a fundamental problem to understanding directed spread of the virus from neurons to innervated tissues. To date, observations of viral assembly in axons have been made in fixed cells. In this report, we present the first study of herpesvirus maturation in living neurons. Separate viral components were simultaneously monitored in axons by fluorescence microscopy, and correlation of the motion of these structures was used to determine if capsids were enveloped during axonal egress. Several approaches were used to simultaneously image capsids and viral envelope structures, and all findings were consistent with maturation occurring in the neural soma and subsequent axonal transport using the secretory pathway.

287

**The Very Large Alpha-herpesvirus Tegument Protein, VP1/2, Co-sediments with Microtubules in an *in vitro* Assay**

G. W. G. Luxton, S. Haverlock, G. A. Smith, S. Rice; Northwestern University Feinberg School of Medicine, Chicago, IL

Alpha-herpesviruses are large, complex DNA viruses that spread throughout the mammalian nervous system by hijacking the endogenous axonal transport machinery of their hosts, allowing the viruses to travel undetected by the immune system. Although it is an important aspect of the infectious cycle, how these viruses interact with and regulate host transport proteins is unclear. Our recent work implicates the viral tegument proteins, located in between the capsid and envelope, in viral transport. We have found that tegument proteins remain associated with capsids as they move within axons sensory neurons during viral entry and egress (*Proc. Natl. Acad. Sci.* USA. 102(16): 5832-5837). We also have shown that the very large tegument protein, VP1/2, is required for microtubule-based capsid transport during viral egress in non-neuronal cells (*Submitted)*. To determine if VP1/2 directly interacts with microtubule motor proteins and therefore serves as a viral effector of capsid transport, co-sedimentation experiments were performed with microtubules (MTs) and extracts from 293 cells transiently expressing a GFP-VP1/2 fusion protein. VP1/2 only sedimented with MTs when dynein was also able to interact with the polymers. This result suggests that VP1/2 may directly tether the dynein to the capsid surface allowing for intracellular alpha-herpesvirus transport.

288

**Two Herpesvirus Kinases Modulate Plus-End Microtubule Transport in the Vertebrate Nervous System**

K. E. Coller,[1] G. T. Shubeita,[2] S. P. Gross,[2] G. A. Smith[1]; [1]Microbiology/ Immunology, Northwestern University, Chicago, IL, [2]Developmental and Cell Biology, University of California, Irvine, CA

Alpha herpesviruses use microtubule motors to transport bidirectionally in axons of the vertebrate nervous system. Targeted axonal transport is essential for the successful establishment of infection in peripheral ganglia (retrograde transport), and subsequent spread to exposed body surfaces following reactivation from latency (anterograde transport). Appropriate targeting of viral particles during infection requires upregulation of the anterograde component during egress, while the retrograde component remains constant during entry and egress. Two serine/threonine kinases, UL13 and Us3, are virally encoded and possess the potential to coordinate axonal transport. The effects of US3 and UL13 on axonal transport were investigated by infecting explanted dorsal root ganglia with fluorescent-tagged virus encoding mutations in these proteins. By live cell fluorescence microscopy, viral particles encoding kinase mutations transported retrograde similar to wild-type viral particles following infection. However, a mutant virus lacking Us3 and UL13 displayed an increase in minus-end motion during anterograde trafficking to axon terminals. Additionally, this mutant exhibited an increase in pauses and transient directional movements. These observations are consistent with the viral kinases regulating efficient anterograde transport by modification of the viral or host transport machinery.

289

**Translokin and Intracellular Trafficking of FGF2**

S. Meunier, C. Bossard, H. Prats, E. Lacazette; IFR 31, INSERM U589, Toulouse, France

FGF2 is one of the prototypes of the fibroblast growth factor family. It is involved in cell proliferation, developmental processes, wound healing, angiogenesis and tumour progression. Five FGF2 isoforms of 18, 22, 22.5, 24, 34K with different amino-termini are synthesised through an alternative translation initiation process. The secreted 18K form lacking an NLS can interact with membrane receptors and be internalized in the cytoplasm. It can also be translocated into the nucleus during the cell cycle. In order to identify proteins that can interact intracellularly with 18K FGF2 a yeast two-hybrid screening using it as bait allowed the identification of a protein called Translokin. Translokin has been recently shown to be involved in the nuclear tranlocation of the 18K form of FGF2. To further characterize the protein complexes involved in the intracellular trafficking of the 18K FGF2 a new yeast two hybrid screening has been made using Translokin as bait. One of the identified proteins is the sorting nexin 6 (SNX6). Initially SNX6 has been shown to interact with the TGFb receptor. These studies localized SNX6 in the cytoplasm where it is thought to target protein to the Trans-Golgi-Network. This makes SNX6 a potential candidate for sorting out FGF2 from the endocytosis vesicles allowing its trafficking to the nucleus. Another protein identified is the RanBPM protein. RanBPM was originally cloned for its ability to bind the small GTPase Ran. A cytoplasmic and nuclear distribution for the protein has been described. Even if the function of RanBPM is still unclear, it could be involved in the nuclear translocation of the 18K FGF2 form through its potential interaction with the Ran/Importin b pathway that mediates cargo import into the nucleus. Thus Translokin can be considered as an adaptor protein for the internalization of the 18K FGF2.

290

**Nuclear and Division Plane Positioning Studied with Optical Tweezers**

I. Tolic-Norrelykke,[1] L. Sacconi,[2] R. Isabel,[1] F. Pavone[2]; [1]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, [2]European Laboratory for Non-linear Spectroscopy, Sesto Fiorentiono (Florence), Italy

Cells of the fission yeast Schizosaccharomyces pombe have a centrally placed nucleus and divide by fission at the cell center. Microtubules are required for the central position of the nucleus, which in turn may determine the position of the division plane. Alternatively, the division plane may be positioned by the spindle, or by a morphogen gradient or a reaction diffusion mechanism. We investigated the role of microtubules in nuclear positioning, and the role of the nucleus in division plane positioning, by displacing the nucleus with optical tweezers. A displaced nucleus returned to the cell center by microtubule pushing against the cell tips. Nuclear displacement during interphase or early prophase resulted in asymmetric cell division, whereas displacement during prometaphase resulted in symmetric division as in unmanipulated cells. Thus our data suggest that the division plane is specified by the position of the nucleus in early prophase. This mechanism in fission yeast differs from that in animal cells, where the division plane is positioned by the anaphase spindle. Since the yeast nucleus is centered by microtubules during interphase but not in mitosis, the establishment of the division plane at the beginning of mitosis may be an optimal mechanism for accurate symmetric division of fission yeast cells.

291

**Nucleus Movement of Shear Polarized Fibroblasts is Mediated by Cdc42 and Microtubules**

J. S. Lee,[1] M. I. Chang,[1] Y. Tseng,[2] D. Wirtz[1]; [1]Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, [2]Chemical Engineering, University of Florida, Gainsville, FL

Nucleus migration is an essential function during nucleus positioning for tissue growth and development in eukaryotic cells. Despite such importance, the molecular regulators of nucleus movement in interphase fibroblasts have not been identified. Here, we demonstrate that the nucleus of Swiss 3T3 fibroblasts subjected to shear flow displays rigid-body movement and rotation, which depends mainly on the integrity of microtubules, not that of F-actin. Bulk depolymerization of F-actin structure caused minor changes in nucleus movement and shape, where as similar depolymerization of microtubules resulted in a more ductile and motile nucleus. Through inactivation of major members of Rho GTPases, well-known mediators of cytoskeleton reorganization, we find that Cdc42, not RhoA nor Rac1, controls the degree of rotation and extent of translational movements of the nucleus. These results establish Cdc42 as a molecular regulator of shear induced microtubule-dependent nucleus movement in Swiss 3T3 fibroblasts. *Mol Biol Cell. 2005 Feb;16(2):871-80

292

**Plasmids Traffic Along Microtubules During Transfections**

E. E. Vaughan, D. A. Dean; Pulmonary and Critical Care, Feinberg School of Medicine, Northwestern Univeristy, Chicago, IL

Little is known about how plasmids move through the cytoplasm to the nucleus during transfections. It has been suggested that the cytoskeleton

impedes free diffusion of DNA, implying that plasmids would be unable to reach the nucleus. Since transfections do work there must be mechanisms by which DNA circumvents cytoplasmic obstacles. One possibility is that plasmids move to the nucleus in a directed fashion using cytoskeletal motors. To address this, adherent, confluent cells were electroporated with a plasmid expressing luciferase. This provides a model for DNA movement not only applicable to electroporation but also to any type of gene therapy where the DNA becomes free in the cytoplasm (e.g. after endosomal escape). We found that perturbation of the actin cytoskeleton by either stabilization or breakdown did not affect the ability of plasmid to be expressed, suggesting that the actin network is not involved in the movement of DNA through the cytoplasm. In contrast, stabilization of the microtubule network increased reporter gene expression approximately five-fold in cultured cells. This suggests that microtubules are involved in transporting plasmids to the nucleus. Microinjections of a GFP-expressing plasmid in the presence of drugs demonstrated that disruption of the microtubule network resulted in drastically decreased expression. An *in vitro* spin-down assay confirmed these results and showed that DNA interacted with microtubules only in the presence of cell extract. Co-injection of anti-dyenin antibodies with a GFP expressing plasmid significantly decreased levels of GFP expression compared to control, suggesting that dynein is involved in this interaction. Taken together these results suggest that free plasmid moves along the microtubule network, via dynein, to reach the nucleus.

293

**Trafficking and Sorting of Vesicles Containing the Bile Salt Transporter Ntcp**

S. Sarkar,[1] E. Bananis,[1] M. S. Anwer,[2] A. W. Wolkoff,[1] J. W. Murray[1]; [1]Anatomy and Structural Biology and Liver Research Center, Albert Einstein College of Medicine, Bronx, NY, [2]Biomedical Sciences, Tufts University School of Veterinary Medicine, North Grafton, MA

Sodium dependent bile salt uptake by $Na^+$/taurocholate cotransporting polypeptide (ntcp) is increased within minutes following stimulation by cAMP, suggesting a readily available intracellular pool. Although it was proposed that intracellular ntcp cycles to and from the plasma membrane on microtubules (MTs), mechanistic details of this trafficking are not known. In the present study, ntcp-containing vesicles were identified in rat liver lysate and purified in an endocytic vesicle-containing fraction that also contained fluorescent vesicles due to pre-injection of liver with Texas red asialorosomucoid (ASOR) for 5 (early) or 15 (late) min before harvesting the liver. Vesicles were flowed into fluorescent microscopy chambers pre-coated with rhodamine-labeled MTs. Antibodies against ntcp, transferrin receptor, Rab11, Rab4, dynein, kinesin I and kinesin II, were used to determine localization on vesicles. Ntcp-containing vesicles bound to MTs and moved bidirectionally following 50μM ATP addition. Motility of these vesicles towards plus and minus-ends of MTs was mediated by kinesin I and dynein based on inhibition of motility by chemical inhibitors and specific antibodies. Ntcp-containing vesicles localized predominantly with recycling endocytic markers, although ~20% were coincident with early ASOR-containing vesicles. There was little colocalization of ntcp with late ASOR vesicles, suggesting that a segregation event occurred. In support of this possibility, ntcp-containing vesicles that colocalized with ASOR underwent fission, with one daughter vesicles enriched in ntcp (>75%) and de-enriched in ASOR (<18%). Fission was not seen in ntcp-containing vesicles that did not contain ASOR. These vesicles localized with Rab11, a marker of recycling endosomes. We conclude that the bile acid acid transporter, ntcp, has three subcellular localizations: plasma membrane, recycling endosomes and early endosomes. Regulation of the trafficking and consequent function of this integral membrane protein may have important consequences regarding the pathobiology of liver disease.

294

**Modulation of Organelle Transport by Intermediate Filaments**

V. I. Gelfand, M. Lakonishok, P. S. Fahrner, A. S. Serpinskaya, Y. Chou, R. D. Goldman; Cell and Molecular Biology, Northwestern University, Chicago, IL

The contribution of intermediate filaments (IF) to organelle transport was studied using cultured *Xenopus* melanophores. Melanophores move pigment organelles (melanosomes) along microtubules and actin filaments toward their periphery in response to high intracellular cAMP and toward the cell center in response to low cAMP. However, in addition to these two types of cytoskeletal filaments, melanophores possess a well developed network of vimentin IF extending from the perinuclear region to the cell surface. In order to investigate the role of IF in the movement and positioning of melanosomes, we transfected melanophores with a construct encoding a GFP-tagged fragment of vimentin including the N-terminus and the first 37 amino acids of the coiled-coil domain 1A. This construct is a dominant-negative disruptor of vimentin IF. The expressed protein was concentrated in perinuclear aggregates and no IF (either endogenous or GFP-tagged) could be detected in the remaining cytoplasm. The expression of GFP-tagged full-length vimentin cDNA, used as a control, assembled into endogenous IF networks that appeared identical to those found in untransfected cells. Comparisons of organelle movement in cells expressing the mutant protein with cells expressing the full-length GFP-tagged vimentin or untransfected cells demonstrated that the destruction of the vimentin IF network substantially increased the rate of movement of melanosomes along microtubules both toward the cell center and toward the cell periphery, but did not affect the final aggregated or dispersed state of pigment. Melanosomes purified from the cell extract by sedimentation through a Percoll cushion contain a substantial amount of vimentin suggesting that pigment organelles can bind to IF. These results suggest that IF play important roles in modulating organelle motility and their anchorage to target sites in the cytoplasm. Supported by grants to VG and RG from the NIGMS.

295

**Spatial and Temporal Dynamics of Vesicle Transport in *Drosophila* Segmental Nerve Axons Revealed by Particle Tracking**

G. Yang,[1] S. B. Shah,[2] G. F. Reis,[2] G. Danuser,[1] L. S. B. Goldstein[2]; [1]Dept. of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Dept. of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA

Proper functioning of microtubule-based bidirectional vesicle transport within nerve axons is crucial for proper neuronal function. Movement of specific cargos can be directly observed through imaging different fluorescently labeled transport proteins. Combination of fluorescence imaging with genetic manipulation of specific components of the transport machinery makes it possible to probe the function and regulation mechanism of the transport process. However, for quantitative understanding and modeling of axonal transport at both single vesicle and ensemble levels, reliable and efficient computational tools must be developed to track vesicle motion and to generate complete spatial and temporal dynamics readouts. Vesicles constantly undergo dense antiparallel motion and crossing, merging, and splitting within the axon, posing a major challenge for computational tracking. We have developed a new particle tracking scheme using a modified optimal bipartite-graph assignment algorithm that is derived from quantitative fluorescent speckle microscopy. A novel graph flow algorithm has been developed to resolve track crossing, merging,

and splitting. Our software can reliably recover complete tracks of large numbers of vesicles and is suitable for high-throughput processing. Based on complete vesicle trajectories, new quantitative descriptors have also been developed to fully characterize the spatial and temporal dynamics of the transport process. We validated this scheme on *in vivo* movies of vesicle motion in *Drosophila melanogaster* larval axons. Movement of kinesin-associated cargoes was observed and measured by imaging expressed YFP fused to the C-terminal of human amyloid precursor protein (APP-YFP) in a subpopulation of *Drosophila* segmental nerves. We report heterogeneity in spatial and temporal dynamics in wild-type larvae. We also report changes in vesicle transport dynamics in larvae with mutation in the retrograde motor component dynein heavy chain.

296

**Regulating Bidirectional Transport: Structure-function Analysis of the Coordinator Klar**

D. Kim,[1] Y. Guo,[1] S. Cotton,[1] M. Zapata,[2] J. G. Gindhart,[2] M. A. Welte[1]; [1]Rosenstiel Center MS029, Brandeis University, Waltham, MA, [2]Biology, University of Richmond, Richmond, VA

Bidirectional transport along microtubules is crucial for many cellular processes, but how opposing motors work together to yield regulated net transport remains unresolved. In *Drosophila*, the Klar protein is important both for transport reversal of certain cargoes and for coordinating motors on individual organelles. Klar's molecular mode of action is unknown. We found that there are at least four distinct Klar isoforms: α, β, γ, δ. Using molecularly characterized alleles, we can disrupt various combinations of isoforms, to probe their developmental expression, intracellular localization, and function. α is necessary for migration of photoreceptor nuclei; β mediates transport of embryonic lipid droplets; δ and γ localize to the nuclear envelope in certain tissues. Comparison with other isoforms suggests that Klar β contains three functionally distinct regions: an N-terminus shared with α, a common core shared with α and δ, and the β-specific C-terminal LD domain. LD is necessary and sufficient to recruit Klar to lipid droplets and thus constitutes a cargo-specific targeting domain. In a yeast-two-hybrid assay, LD interacts with LSD2, a droplet-specific transport regulator. LSD2 is not a droplet receptor for LD, or vice versa, because LSD2 and Klar are recruited to lipid droplets independently of each other. For two additional proteins that interact with LD in yeast, we are currently testing for physical interactions with LD in vivo and in vitro and are analyzing phenotypes of single and double mutants. We propose that the β-specific LD domain not only mediates correct intracellular localization of Klar but also confers cargo-specific regulatory properties to Klar. As a C-terminal LD fragment is sufficient to target to droplets in cultured cells, droplet localization and binding of regulators may be mediated by distinct regions of LD.

297

**Hic-5, a Focal Adhesion LIM Protein, Organizes a Nuclear-cytoplasmic Shuttling Complex Through LIM-LIM Recognition Involved in Cell-cycle Control**

K. Mori, K. Nose, M. Shibanuma; Dept. of Microbiology, Showa University, Tokyo, Japan

Hic-5 is a protein most homologous to paxillin, consisting of four N-terminal LD and four C-terminal LIM domains, and along with serving as a scaffold for signaling at focal adhesions, it shuttles between the focal adhesions and the nucleus. We report here the unique property of the most C-terminal LIM domain of Hic-5 to dimerize and the identification of the new partners of Hic-5 that heterodimerize and communicate between the two compartments with Hic-5. The biological significance of the complex formation is also addressed. RESULTS: Biochemical and immunoprecipitation study showed that Hic-5 formed homodimers as well as heterodimers with two LIM-only scaffold proteins, CRP and PINCH, in marked contrast to paxillin. Hic-5 also interacted with ILK, suggesting the assembly of multiprotein signaling complexes including ILK based on docking of the two scaffold proteins, Hic-5 and PINCH. Further study showed that the interaction of Hic-5 and PINCH was mediated by their particular single LIM domains, and both proteins were primarily co-localizaed at focal adhesions. However, under leptomycin B (LMB) treatment, PINCH, similar to Hic-5, accumulated in the nucleus when co-expressed with Hic-5. This phenomenon was also dependent on the co-expression of ILK whose location remained in the cytoplasm. These results, together with the study using various mutants defective in the interaction and shuttling, suggested that the interaction with Hic-5 directed PINCH to shuttle between cytoplasm and the nucleus, depending on ILK in the cytoplasm. Similarly, CRP showed LMB-dependent nuclear accumulation in the presence of Hic-5 or else localized in actin stress fibers in the cytoplasm. When introducing interaction-defective mutants of Hic-5 and PINCH into cells, the inhibition of cell-cycle entry and BrdU incorporation was observed. The detailed mechanisms are now under investigation.

298

**Fbox8, a Fbox Protein with the Sec7 Domain, Makes Arf6 Refractory to Function via Ubiquitination**

H. Yano,[1] I. Kobayashi,[1,2] K. Miura,[1] K. Iwai,[3] Z. Ronai,[4] H. Sabe[1,2]; [1]Department of Molecular Biology, Osaka Bioscience Institute, Suita, Japan, [2]Graduate School of Biostudies, Kyoto University, Kyoto, Japan, [3]Department of Molecular Cell Biology, Graduate School of Medicine, Osaka City University, Osaka, Japan, [4]Signal Transduction Program, The Burnham Institute, La Jolla, CA

The small GTP-binding protein Arf6 plays pivotal roles in membrane and cytoskeletal remodeling at the cell periphery, and has been implicated in higher orders of the cellular functions. Here we show that Arf6 can be polyubiquitinated, and identified Fbox8, an Fbox protein bearing the sec7 domain, to be responsible for this ubiquitination. Arf6 ubiquitination occurred predominantly at particulate subcellular fraction. No clear evidence was obtained for immediate degradation of the ubiquitinated Arf6, while Fbox8 was also ubiquitinated and rapidly degraded via proteasome in the same sets of cells. We also show that Fbox8 is predominantly recruited to plasma membrane rufflings where Arf6 is expected to function upon EGF stimulation, and suggest that ubiquitinated Arf6 molecules may escape from being recognized by its regulators and effectors such as ARNO and AMAP1. Forced expression of Fbox8 blocked cancer cell invasion and receptor endocytosis. We propose that Fbox8 provides a novel mechanism of Arf6 regulation.

299

**Akt Mediates Growth Factors Induced Cell Motility through Increasing Serine Phosphorylation of Phospholipase C-γ1**

Y. Wang, Z. Wang; Cell Biology, University of Alberta, Edmonton, AB, Canada

Both Phospholipase C-γ1 (PLC-γ1) and Akt (Protein kinase B, PKB) are signaling proteins that play significant roles in the intracellular signaling mechanism utilized by receptor tyrosine kinases (RTKs) including epidermal growth factor (EGF) receptor (EGFR). EGFR can activate PLC-γ1 directly and can activate Akt indirectly through PI3K. Many data have shown that PLC-γ1 pathway and PI3K-Akt pathway interact each other.

However, it is not known whether PLC-γ1 binds to Akt and what is the function of this interaction. In this communication, we identified a novel interaction between PLC-γ1 and Akt. We demonstrated that the interaction is mediated by the binding of PLC-γ1 SH3 domain to Akt proline motifs. We also provide a novel model to depict how the interaction between PLC-γ1 SH3 domain and Akt proline motifs is dependent on EGF stimulation. In this model, phosphorylation of PLC-γ1 Y783 by EGF causes the conformational change of PLC-γ1 to allow the interaction of its SH3 domain with Akt proline motifs. Furthermore, we showed that the interaction between PLC-γ1 and Akt caused phosphorylation of PLC-γ1 S1248 and enhanced EGF-stimulated cell motility.

300

**Anomalous Diffusion in Living Cells**

M. Elsner,[1] M. Weiss,[2] T. Nilsson[1]; [1]Medical Biochemistry, Göteborg University, Göteborg, Sweden, [2]BIOMS, Group Cellular Biophysics, German Cancer Research Center, Heidelberg, Germany

Since the advent of advanced light microscopy techniques it has become possible to investigate diffusion properties of molecules in living systems and to exploit these properties to gain insight in the organisation of cells. For a long time the basic physico-chemical properties of the cytosol received little attention. Only since the late 90s it is widely recognized that the high concentration of macromolecular solutes in the cytosol lead to properties qualitatively different from an average buffer solution. This phenomenon has been termed 'molecular crowding'. It influences basic cellular processes like protein folding, enzyme kinetics and DNA condensation. Here, we investigated the diffusion properties of fluorescently labelled inert dextran polymers in the cytosol of living cells with fluorescence correlation spectroscopy. Using dextrans of different molecular weights we found that all show a size dependent anomalous subdiffusion in the cytosol of living cells. In anomalous subdiffusion the mean square (MSD) displacement of the diffusing particle does not grow linear with time as in normal diffusion, but MSD~ $t^{\alpha}$ with $\alpha<1$. We could rule out that higher order structures like the cytoskeleton or the Endoplasmic Reticulum cause the observed subdiffusion. In contrast, we could demonstrate that a high concentration of macromolecular solutes leads to the emergence of anomalous diffusion using computer simulations and *in vitro* measurements. The extent of the anomality depends of the concentration and sized distribution of the solutes, which should make it possible to use this property to define the extent of crowding in a given solution, e.g. the cytosol.[1] Anomalous diffusion is not only observed in the cytosol but is also observed on Golgi resident transmembrane proteins. All Golgi resident proteins investigated so far show anomalous subdiffusion, suggesting a complicated organisation of Golgi membranes. 1 Weiss M, Elsner M *et al.* Biophys J, (2004)

301

**Mitochondrial Transport and Location Within Axons Are Not Associated with Differences in Membrane Potential**

J. L. Verburg,[1] R. L. Morris,[2] P. J. Hollenbeck[1]; [1]Biological Sciences, Purdue University, West Lafayette, IN, [2]Biology, Wheaton College, Norton, MA

The transport and docking of mitochondria in neurons place them at sites in the cell presumed to have a high demand for ATP and/or calcium buffering. Once mitochondria are directed toward an appropriate region, the cell could further increase local ATP production by locally increasing mitochondrial membrane potential ($\Delta\Psi$). Using the dye JC-1 as a reporter, we found that mitochondria moving anterogradely had higher apparent $\Delta\Psi$ than those moving retrogradely, in agreement with published data (Miller and Sheetz, 2004). However, since JC-1 has been shown to form irreversible aggregates providing an overestimate of $\Delta\Psi$, we made in vivo measurements using the Nerstian dye tetramethylrhodamine methyl ester (TMRM), which equilibrates rapidly across the membrane and does not aggregate. Mitochondrial TMRM fluorescence is diminished by uncoupling agents and increased by inhibition of ATP synthase. Fluorescence values from mitochondria were collected and compared between different regions of the axon. Although TMRM reveals heterogeneity in mitochondrial $\Delta\Psi$, the intracellular variation is surprisingly small. The standard deviation in $\Delta\Psi$ among mitochondria within individual neurons is 3.5 times less than that found when mean $\Delta\Psi$ of mitochondria was compared among different cells. To assess possible regional differences in $\Delta\Psi$, mitochondrial TMRM fluorescence was compared among three regions of the axon: growth cones, branch points, and axonal shafts. No correlation was found between mitochondrial fluorescence and the location of mitochondria in the axon. Comparing the $\Delta\Psi$ of anterograde, retrograde, and non-moving mitochondria revealed no significant difference among these populations. The lack of intracellular variation of $\Delta\Psi$ when measured with TMRM suggests that growing neurons use global rather than local cues to control $\Delta\Psi$, and rely on the physical distribution of mitochondria to serve the needs of specific subcellular regions.

302

**Hsp90 Chaperon Activity Regulates GDI Function in Rab1 Recycling Pathway**

C. Y. Chen,[1] W. E. Balch[2]; [1]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Department of Cell Biology, Molecular Biology and the Institute for Childhood and Neglected Disease, The Scripps Research Institute, La Jolla, CA

Rab GTPase-regulated hubs directing vesicle tethering-fusion provide the framework for an integrated coding system, the *membrome* network (Gurkan et al. (2005) Mol. Biol. Cell, 16, 3847), which is responsible for generation of the membrane architecture of eukaryotic cells. Guanine nucleotide dissociation inhibitor (GDI) is a central component of the Rab GTPase cycle that retrieves prenylated Rab GTPases from the lipid bilayer to form a cytosolic GDI-Rab complex. We have previously shown that Hsp90 specific inhibitors, such as geldanamycin (GA) and radicicol, potently inhibit αGDI-dependent recycling of Rab3A at the synapse and neurotransmitter transmitter release (Sakisaka et al. (2002) EMBO J., 21, 6125). We now show that the recycling of the Rab1 GTPase involved in constitutive transport between the ER and the Golgi is regulated by an Hsp90 complex. We find that GA and radicicol prevent the ability of GDI to retrieve Rab1 from the Golgi membranes. Inhibition of Hsp90 activity targets Rab1 function, which blocks ER to Golgi transport and prevents Golgi re-assembly. It is well established that Hsp90 chaperon complex modulates the conformation of steroid hormone receptor (SHR) to bind its substrate, steroid hormone. The similarity between SHR/steroid hormone and GDI/Rab prenyl groups suggests that membrane-associated Hsp90 chaperone complex alters the structure of the recruited GDI to form a lipid-binding pocket that facilitates the retrieval of Rab prenyl groups from the lipid bilayer for recycling to the cytosol.

303

**Microtubule Acetylation Inhibits Directional Migration, Cellular Polarization and Bidirectional Vesicle Transport**

A. A. Salam,[1] D. A. Recinos,[1] T. P. Yao,[2] J. C. Bulinski[1]; [1]Biological Sciences and Pathology & Cell Biology, Columbia University, New York,

NY, [2]Pharmacology and Cancer Biology, Duke University, Durham, NC
During persistent directional migration, a cell orients intracellular organelles and vesicle trafficking towards its leading edge. Several studies have implicated the activity or level of a MT-selective deacetylase, HDAC6, or the level of acetylated (Ac) MTs in regulating cell motility and polarizing cells (Hubbert et al., Nature 417:455-458[2002]; Haggarty et al., PNAS 100:4389-4394[2003]; Serrador et al., Immunity 20:417-428[2004]). To test the hypothesis that increased MT acetylation (hyper-Ac MTs) limits directional motility by inhibiting cellular polarization, we utilized trichostatin A (TSA) to inhibit HDAC6 activity, thus acutely increasing Ac MTs. In wound-healing assays TSA treatment significantly decreased directional migration, concomitantly decreasing the polarization of four organelles, the MT organizing center (MTOC), the stable MT subset, the Golgi complex, and the vimentin network. We next tested whether HDAC6-inhibition is sufficient to hamper migration of wound-edge cells that had already polarized prior to TSA treatment; preliminary data show that increasing Ac MTs in pre-polarized cells inhibits velocity of migration and mislocalizes the MTOC and the Golgi complex. TSA-inhibited HDAC6 localizes to MTs in the presence of TSA (A.Tran, not shown), thus, we tested whether our results are attributable to hyper-Ac MTs or MT-bound, inhibited HDAC6, using cell lines deficient in HDAC6. Preliminary experiments showed that siRNA HDAC6 knockdown cells were markedly defective in directional motility. We next tested whether the level of Ac MTs or HDAC6 alters Golgi vesicle trafficking; polarization of the Golgi complex is known to contribute to directed cell migration. Consistent with our hypothesis, TSA treatment resulted in a significant decrease in bi-directional vesicle trafficking, as did lowering HDAC6 expression. Our results point to a possible mechanism by which Ac MTs may affect directional motility, and our data underscore the importance of this MT subset in modulating MT functions.

## Intermediate Filaments I (304-323A)

304

### Association of Keratin 8 but not Keratin 19 Variants with Inflammatory Bowel Disease

G. Tao,[1] Q. Zhou,[1] P. Strnad,[1] L. Zhang,[2] N. D. Madani,[1] S. R. Brant,[3] J. Cho,[4] M. B. Omary,[1] R. H. Duerr[2]; [1]Department of Medicine, Palo Alto VA and Stanford University, Palo Alto, CA, [2]Department of Medicine and Human Genetics, University of Pittsburgh, Pittsburgh, PA, [3]Department of Medicine, Johns Hopkins University, Baltimore, MD, [4]Department of Medicine, University of Chicago, Chicago, IL
Keratin 8 (K8)-null mice develop spontaneous colitis, and K8 variants are found in few patients with ulcerative colitis (UC) and Crohn's disease (CD) but the association did not reach statistical significance. We asked if mutations in K8 or K19, the major intestinal keratins, predispose to UC or CD. Exonic K8 and K19 regions were PCR-amplified using genomic DNA from 87 unrelated patients with CD, 100 patients with UC, and 70 unaffected volunteers. Mutations were analyzed using a WaveSystem™ and DNA sequencing. Presence of K8 variants was tested by Pyrosequencing™ in 401 nuclear families (396 independent families with 667 UC/CD-affected offspring) that contain both parents and at least one UC/CD-affected offspring. K19 variants were identified in UC/CD patients with a similar mutation frequency in the control group. In contrast, 16 of the 187 UC+CD patients harbored K8 heterozygous variants involving G61C (6 patients) and R340H/C (11 patients, including one compound heterozygous) that were not found in any of the volunteers ($p<0.03$). The R340→C variant is unique and has not been previously described. The number of heterozygous parents and minor allele frequencies for each variant in the nuclear family parents are G61C (11; 1.4%), R340C (1; 0.13%), and R340H (33; 4.1%). Transmission disequilibrium testing showed transmissions:nontransmissions=6:1 (CD) and 7:4 (UC) for G61C, 0:1 (CD) and 2:0 (UC) for R340C, and 19:13 (CD) and 10:13 (UC) for R340H and correlation with disease severity is being analyzed. Hence, K8 but not K19 variants are found at higher frequency in patients with UC and CD and may play a role in disease pathogenesis. Establishment of significant transmission disequilibrium will require a larger cohort. K8 variants could pose an increased risk for developing UC/CD or end-stage liver disease depending on additional genetic/environmental factors.

305

### Interaction Between the Intermediate Filament Protein Synemin and α-Actinin in Mammalian and Avian Muscle Cells

S. E. Veneziano,[1] R. C. Bhosle,[1] Z. Li,[2] D. Paulin,[2] R. M. Robson[1]; [1]Muscle Biology Group, Iowa State University, Ames, IA, [2]Biologie Moleculaire de la Differenciation, Universite Paris, Paris, France
Intermediate filaments (IFs) encircle skeletal muscle myofibrils at their Z-lines, thereby linking all adjacent myofibrils, and the Z-lines of the peripheral layer of myofibrils to costameres along the sarcolemma. Synemin is a large novel member of the IF protein superfamily and appears to play a critical cross-linking role within the muscle cell cytoskeleton. Avian synemin exists as one form, but at least two splice variants, a larger α-synemin (ortholog of avian synemin) and a smaller, β-synemin, are differentially expressed in mammalian skeletal and smooth muscle. Synemin forms heteropolymeric IFs with the major IF proteins desmin and/or vimentin. Previous experiments demonstrated that avian synemin binds to α-actinin, a major component of both skeletal muscle Z-lines and smooth muscle dense bodies. It is not clear whether functional differences exist between α- and β-synemins, or whether one or both of these mammalian isoforms interact with α-actinin. Our objectives were to determine if an interaction exists between synemin and α-actinin in mammalian skeletal and smooth muscle cells, to define the interaction sites within each protein, and to compare this interaction to that in avian skeletal muscle cells. Blot overlay experiments indicate the C-terminal tail region of α-synemin binds to both head and rod domains of α-actinin-2, whereas β-synemin does not. Co-immunoprecipitation studies indicate synemin and α-actinin interact within cultured mouse skeletal muscle cells, rat smooth muscle cells, and primary chick skeletal muscle cells. In eight-day primary chick skeletal muscle cell cultures, synemin organizes into a striated pattern and colocalizes with α-actinin. Our results indicate that mammalian α-synemin, but not β-synemin, interacts with α-actinin in both skeletal and smooth muscle, thereby playing an important role in cytoskeletal linkages in mammalian skeletal and smooth muscle cells. (Supported by USDA-CSREES-NRICGP Award 2003-35206-12823)

306

### Interactions of Two Isoforms of Mammalian Synemin with Vinculin and Metavinculin

N. Sun,[1] Z. Li,[2] D. Paulin,[2] D. R. Critchley,[3] R. M. Robson[1]; [1]Muscle Biology Group, Iowa State University, Ames, IA, [2]Biologie Moléculaire de la différenciation, Universite Paris, Paris, France, [3]Department of Biochemistry, University of Leicester, Leicester, United Kingdom
Synemin is a unique member of the intermediate filament (IF) protein superfamily, and is present as two splice variants, α and β, in mammalian muscle cells. The larger α-synemin is considered an ortholog of avian muscle synemin, which is expressed as only one form. Neither avian

synemin nor the two mammalian synemin isoforms will form IFs by themselves, requiring other IF proteins such as desmin or vimentin to form heteropolymeric IFs. The heteropolymeric IFs surround the Z-lines of the myofibrils in striated muscle cells, and thereby link all adjacent myofibrils within the cell. The IFs also link the Z-lines of the peripheral layer of myofibrils to the costameres positioned periodically along and subjacent to the sarcolemma. The costameric protein vinculin was previously shown to interact with avian synemin. Human α-synemin exhibits approximately 35% sequence identity with its avian ortholog. Solid-phase protein binding assays (blot overlays) and GST pull-down assays reveal specific interaction of the human vinculin tail domain with human α-synemin, but not with the β-synemin isoform. The region of interaction is located within the very long C-terminal tail domain of α-synemin. Immunocytological staining of cultured rat smooth muscle cells shows co-localization of α-synemin and vinculin in both focal adhesions and membrane ruffles. Protein binding assays also reveal that α-synemin interacts with the tail domain of human metavinculin, which is a slightly larger isoform of vinculin. In GST pull-down assays, vinculin and metavinculin display somewhat differential binding characteristics for α-synemin. These results suggest the possibility of isoform-specific functional roles for α and β synemin and for vinculin and metavinculin within mammalian muscle cells. (Supported by USDA-CSREES-NRICGP Award 2003-35206-12823)

307

### A Novel Mutation Reveals a Role of Keratin 5 in Melanosome Distribution and Cell Shape

L. Planko,[1] R. Betz,[2] S. Pasternack,[2] R. Kruse,[3] H. Buessow,[4] T. M. Magin[1]; [1]Institut für Physiologische Chemie, Abteilung für Zellbiochemie and LIMES, Universität Bonn, Bonn, Germany, [2]Institut für Humangenetik, Universität Bonn, Bonn, Germany, [3]Dermatologische Klinik, Universität Düsseldorf, Düsseldorf, Germany, [4]Anatomisches Institut, Universität Bonn, Bonn, Germany

Epidermal fragility syndromes support the concept that keratins have a major role in protecting epithelia against mechanical trauma. Dominant mutations in K5 and K14 cause EBS, a disease of basal epidermis accompanied by cytolysis and aggregation of the keratin cytoskeleton. Recent observerations have indicated an additional function of keratins in signal transduction. This also raises the issue whether intermediate filament proteins function both in their insoluble and a soluble state and how the transition between the two states is regulated. Here, we describe a novel, dominant K5 mutation which does not cause aggregation of the keratin cytoskeleton but alters tissue morphology characterized by papillary downgrowth of the epidermis. Moreover, it affects melanosome distribution in keratinocytes. The insertion of a single nucleotide causes premature translation termination which encodes a shortened K5 polypeptide encompassing the head domain. By RT-PCR, we demonstrated the presence of the mutant mRNA, excluding nonsense-mediated decay. Upon stable transfection as a YFP fusion protein into cultured keratinocytes, the truncated YFP-K5 polypeptide displayed a diffuse cytoplasmic localization and was completely soluble following fractionation. Colocalization studies indicated no association with desmoplakin, unliked predicted by previous studies. Instead, we noted a partial colocalization with dynein intermediate chain which may be involved in melanosome transport in keratinocytes. By coimmunoprecipitation, we isolated candidate proteins interacting with the K5 head domain. We hypothesize that the soluble keratin fraction is strictly regulated and has a major role in melanosome transport and localization.

308

### A Supportive Role of Desmin in Early Cardiomyogenesis

M. Hofner, A. Höllrigl, S. Puz, M. Stary, G. Weitzer; Medical Biochemistry, Medical University Vienna, Vienna, Austria

Desmin contributes to the stability of the myocardium and mutant alleles co-segregate with cardiomyopathies. To understand the role of desmin in cardiomyogenic specification and differentiation we investigated early cardiomyogenesis in embryoid bodies made of embryonic stem cells expressing different *desmin* alleles. We demonstrated a supportive role of desmin during mesoderm specification and early differentiation of cardiomyocytes, and an important function of amino-terminally located and facultative phosphorylated serine residue in specification, differentiation and maintenance of the myocardial phenotype. Constitutive expression of *desmin* assisted early cardiomyogenesis by temporary upregulation of *brachyury*, *nkx2.5*, *mlc1v* and *mhcα*. Consecutive development of highly interconnected and synchronously beating cardiomyocyte clusters pointed at an improved cytodifferentiation of cardiomyocytes. Desmin null cardiomyocytes featured an opposite phenotype. Constitutive expression of amino-terminally truncated $desmin^{\Delta 1\text{-}48}$ interfered with cardiomyogenesis, induced early cell death of presumptive cardioblasts, caused temporary downregulation of mesodermal and myocardial transcription factors, and hampered myofibrillogenesis and survival of cardiomyocytes. Mutation of temporarily phosphorylated serine residues in the amino-terminal domain of desmin destructed cardiomyogenesis and myofibrillogenesis in a dominant negative fashion. Even before beating cardiomyocytes developed $desmin^{S6,7,8A}$ caused downregulation of the transcription factor genes *brachyury*, *goosecoid*, *nkx2.5* and *mef2C*, which was accompanied by increased apoptosis of presumptive cardioblasts. $Desmin^{S31,32A}$ attenuated cardiomyocyte proliferation and cell-cell interaction, however did not affect early cardiomyogenesis and expression of mesodermal and myocardial transcription factors. These results demonstrate the detrimental influence of mutated desmin on specification and cytodifferentiation of early cardiomyocytes and suggest a substantial involvement of desmin in the very beginning of cardiomyogenesis. This work was supported by grant from Austrian FWF and bm:bwk.

309

### Mechanism of Keratin Filament Remodeling under Flow in Cultured Human Alveolar Epithelial Cells

F. W. Flitney,[1] K. M. Ridge,[2] R. D. Goldman[1]; [1]Cell & Molecular Biology, Northwestern University, Chicago, IL, [2]Division of Pulmonary & Critical Care Medicine, Northwestern University, Chicago, IL

Fluid shear stress results in a restructuring of keratin IF in cultured lung epithelial cells (Ridge et al, JBC In Press). The mechanism underlying the response to flow has now been studied using human alveolar cells (A549). Cells stained with anti-K18 were found to contain numerous densely-packed, spherical particles distributed throughout the cytoplasm, masking the underlying IF network. However, a well-developed keratin IF network consisting of thin, predominantly straight tonofibrils was revealed when cells were fixed in methanol, which did not preserve the fluorescent particles. This suggests that the latter might represent a more soluble pool of non-polymerized keratin. Immunoblots demonstrated that K18 was present in the soluble fraction following extraction with TX-100 in IF stabilization buffer. When A549 cells were subjected to moderate fluid shear stress (7 dynes/cm$^2$, 2-6 h), the majority of the fluorescent particles disappeared and their loss correlated with a >95% decrease in the amount of K18 in the TX-100 soluble fraction. Furthermore, the thin bundles of IF were transformed by flow into much thicker, wavy tonofibrils

that were readily seen by both immunofluorescence and TEM. We conclude that A549 cells contain a significant 'pool' of soluble K18, estimated to be ~10-12% of the total amount present in the cell. Our results show that shear stress creates conditions that facilitate the incorporation of this soluble fraction into pre-existing IF, an adaptive response to flow that results in a more robust, mechanically-stable IF network. We suggest that the reconfigured network is stiffer and therefore better able to resist potentially damaging mechanical forces, such as those generated during mechanical ventilation of the fluid-filled, atelectatic lung in patients with acute lung injury. Supported by a grant from the National Heart, Lung and Blood Institute.

310

**Plectin-Controlled Intermediate Filament Cytoarchitecture Affects MAP Kinase-Mediated Stress Response and Migration of Cells**

S. Osmanagic-Myers, M. Gregor, G. Walko, S. Reipert, G. Wiche; Molecular Cell Biology, University of Vienna, Vienna, Austria

Plectin is a major intermediate filament (IF)-based cytolinker protein capable to interact with a variety of cytoskeletal structures and plasma membrane-bound junctional complexes. Beyond stabilizing cells and tissues mechanically, plectin regulates actin filament dynamics and serves as a scaffolding platform for signaling molecules. In this study we show that plectin deficiency is a cause of aberrant keratin and vimentin cytoskeleton organization due to a lack of orthogonal IF-crosslinking. Keratin networks in plectin-deficient cells were found to be more susceptible to osmotic shock-induced retraction from peripheral towards perinuclear areas. Similarly, okadaic acid-induced disruption of IFs, both in fibroblasts and keratinocytes, proceeded much faster when plectin was absent. In both cases, accelerated stress response of IFs was paralleled by faster activation kinetics of stress-activated MAP kinase p38. In contrast, already the basal activity of migration-controlling MAP kinase Erk2 was significantly elevated in plectin-deficient keratinocytes correlating with their increased migration potential as assessed in vitro. Our data establish a direct link between cytolinker-controlled IF cytoarchitecture and specific MAP kinase cascades mediating distinct cellular responses.

311

**Elastic and Plastic Behaviour of the Intermediate Filament Network in Keratinocytes**

D. S. Fudge,[1] D. Russell,[2] E. B. Lane,[2] A. W. Vogl[3]; [1]Integrative Biology, University of Guelph, Guelph, ON, Canada, [2]Cancer Research UK Cell Structure Research Group, University of Dundee, Dundee, United Kingdom, [3]Cellular and Physiological Sciences, Univ. of British Columbia, Vancouver, BC, Canada

Recent work on the properties of secreted intermediate filaments (IF) from hagfish suggest that bundles of IFs are much less stiff and far more extensible than previously believed. We tested whether the mechanical properties of IF bundles in cultured cells are consistent with the properties reported from secreted hagfish IFs. To do this, we constructed a custom stretching apparatus that allowed us to apply uniaxial strains of up to 150% to NEB K14-GFP human keratinocytes grown on silicone rubber membranes. This apparatus was mounted on a fluorescent microscope so that the IF network could be visualized as the cells were stretched. We used a similar apparatus to embed stretched cells for visualization using TEM. Our light microscopy results showed that the IF network survives cell strains up to 150% without obvious rupture. At low cell strains, IF bundles behaved elastically. At higher strains, however, deformation was plastic, i.e. IF bundles did not recover to their initial length when the stretching protocol was reversed. TEM images of stretched and control cells revealed that low uniaxial strain results primarily in a straightening of IF bundles, which tend to be wavy in unstretched cells. Higher strains resulted in more direct longitudinal loading of the IF bundles, with plastic behaviour likely arising from slippage between IFs in the bundle, or possibly even stretching of the IFs themselves. The remarkable extensibility and mechanical stability of the IF network demonstrated here suggest that the IF network functions to passively protect cells and tissues from dramatic stresses and/or deformations.

312

**Crescentin Imitates Cytoplasmic Intermediate Filaments *in vitro* and is Temporally Regulated *in vivo***

M. T. Cabeen,[1] N. Mücke,[2] H. Herrmann,[3] C. Jacobs-Wagner[1]; [1]Molecular, Cellular & Developmental Biology, Yale University, New Haven, CT, [2]Biophysics of Macromolecules, German Cancer Research Center, Heidelberg, Germany, [3]Cell Biology, German Cancer Research Center, Heidelberg, Germany

Crescentin is the first known bacterial homologue of an intermediate filament (IF) protein, and is required for the curved-rod morphology of *Caulobacter crescentus*. It localizes in wild-type cells to a prominent filamentous structure at their inner curvatures, consistent with a cytoskeletal function. We sought first to compare the *in vitro* biochemical properties of crescentin with those of eukaryotic IF proteins. We used analytical ultracentrifugation to show that purified wild-type crescentin exists as a soluble tetramer, similar to eukaryotic IF proteins such as vimentin. In the presence of magnesium ions, crescentin formed filamentous structures similar in appearance to eukaryotic IF. These results suggest that crescentin follows an assembly pathway similar to those of cytoplasmic IF proteins such as desmin and vimentin. Considering that cytoplasmic IF proteins are generally considered to have evolved from lamin-like IF proteins, these results are particularly interesting. To explore crescentin regulation in live cells, we took advantage of the genetic tractability of our bacterial system and made a strain bearing a xylose-inducible *creS* gene. Surprisingly, we found that induced expression of *creS*, rather than complementing the straight phenotype of a strain deleted for *creS*, slowed growth and caused division defects. To further explore this phenotype, we used fluorescence imaging of a strain bearing an inducible tetracysteine (TC)-tagged version of crescentin, which was specifically labeled with Lumio Green dye. This revealed that xylose-induced crescentin forms a thick, mislocalized filamentous structure that appears to prevent cytokinesis. However, when crescentin is overexpressed in a strain with a high-copy plasmid bearing *creS* and its native promoter, cells exhibit significantly increased curvature. This suggests that increased expression of crescentin increases curvature, but only when crescentin expression is temporally regulated by its promoter.

313

**Integrin β3 Mediates Interaction of Vimentin with Focal Contacts in Endothelial Cells**

R. Bhattacharya, J. C. Jones; Cell and Molecular Biology, Northwestern University, Chicago, IL

We have previously demonstrated that vimentin-type intermediate filaments (IFs), in addition to microfilaments, associate with αvβ3 integrin-positive focal contacts in endothelial cells. Moreover, we have provided evidence that vimentin regulates the size, dynamics and adhesive functions of focal contacts (Tsuruta and Jones, 2003). In this study we have investigated the role of integrins in mediating vimentin-focal contact interaction.

Knocking down β3 integrin expression levels in endothelial cells using siRNA results in up to a 70% decrease in the numbers of focal contacts showing association with IF. Moreover, when endothelial cells are plated on fibronectin-coated coverslips, focal contacts are depleted of β3 integrin and we again observe a dramatic decrease in vimentin-focal contact interaction. To provide further support for β3 integrin-mediated vimentin-focal contact interaction, we next transfected GFP-β3 integrin into cell lines lacking β3 integrin but containing vimentin. Specifically, in "parental" CHO cells the vimentin IF cytoskeleton is collapsed around the perinuclear space. In sharp contrast, in CHO cells in which we induced the expression of full-length β3 integrin (CHOwtβ3), vimentin fibers extend to β3 integrin-rich focal contacts at the cell periphery. We have also transfected GFP-vimentin into a cell line lacking vimentin but which expresses β3 integrin (MCF-7 human breast adenocarcinoma cells). In the transfected MCF-7 cells, vimentin squiggles and short vimentin fibers arise from peripheral focal contacts suggesting the intriguing possibility that focal contact sites may be involved in filament nucleation. These data provide evidence that β3 integrin mediates vimentin association with focal contact proteins and we suggest that the latter interaction plays an important role in regulating both focal contact dynamics and functions.

314

**The Production and Distribution of Hsp27 and Keratins Are Affected in Keratinocytes Subjected to Mechanical Stress**

O. Duverger,[1,2] M. Moreau,[1,2] S. Schnebert,[1] C. Nizard[1,2]; [1]R&D LVMH Parfums et Cosmetiques, Saint Jean de Braye, France, [2]INSERM U505, Paris, France

The skin is constantly exposed to various internal and external forces resulting in different kinds of strains. Intermediate filaments are known to be essential for cell resistance to mechanical stress. In the epidermis, different keratins make up the intermediate filament network, according to the cell layer considered. The basal layer contains keratins 5 and 14, whereas the suprabasal layers contain keratins 1 and 10. On the other hand, the small heat shock protein HSP27 is known to protect the cytoskeletton (actin and intermediate filaments) against a large range of stresses. HSP27 can also be specifically involved in the reorganization of intermediate filament networks. In normal epidermis, HSP27 is produced in the suprabasal layers. Thus, we wondered if HSP27 could protect keratinocytes against mechanical stress by interacting with keratins. We analyzed the dynamics of HSP27 and intermediate filaments production and distribution in keratinocytes subjected to cyclic stretching. Stretching results in a rapid and transient reduction of the amount of HSP27 protein. Immunocytochemical analyses confirm this observation and show that HSP27 is progressively produced in groups of cells before getting back to a broad distribution. Interestingly, the distribution of basal keratins follows the same changes as HSP27, with a stricking colocalization of the signals. These results show that stretching alters the organization of keratin networks and suggest that HSP27 is involved in these modifications.

315

**P38 MAPK-dependent Shaping of the Keratin Cytoskeleton**

S. Wöll, R. Windoffer, R. E. Leube; Department of Anatomy and Cell Biology, Johannes Gutenberg University, Mainz, Germany

We recently observed that the tyrosine phosphatase inhibitor vanadate induces rapid and reversible reorganization of the keratin cytoskeleton. It was further noted that these changes were almost completely prevented by prior exposure to light of different wavelengths and, to a lesser degree, by pre-treatment with the p38 MAPK inhibitor SB203580. To further follow up on the latter observation, we examined the phosphorylation of a potential p38 MAPK target site in keratin 8. Less than 30 seconds after exposure to vanadate a strong increase in phosphorylation occurred in this site in the dark that was completely blocked in the light. Although p38 MAPK activation was only mildly affected in these situations as judged by its phosphorylation status, a significant recruitment of phosphorylated p38 MAPK to the keratin system and, especially, newly forming granular aggregates was evident within two minutes of vanadate exposure in the dark. Similar phenomena were noted in other situations of keratin filament restructuring. To directly assess the importance of p38 MAPK for the keratin system shRNA-encoding constructs were prepared. Transfection of these constructs into epithelial cells resulted in significant alterations of the intermediate filament cytoskeleton. Taken together, we propose that p38 MAPK regulates keratin filament formation, most likely by phosphorylation of specific sites.

316

**Cell Stress is a Key Factor in the Phenotypic Variation of the R416W GFAP Mutation Causing Alexanders Disease**

S. Wen,[1] M. Perng,[1] R. Li,[2] M. Brenner,[2] R. A. Quinlan[1]; [1]School of Biological Sciences, University of Durham, Durham, United Kingdom, [2]Department of Neurobiology and Civitan International Research Center, University of Alabama at Birmingham, Birmingham, AL

Alexander Disease (AD) is a fatal neurological disorder characterised by the presence of cytoplasmic inclusion called Rosenthal fibers, which contain astrocytic intermediate filament (IF) glial fibrillary acidic protein (GFAP). Many AD patients contain one of a series mutations in the GFAP gene. In this study, we present evidence to suggest that one of the mutation, R416W , in GFAP requires additional factors to precipitate AD. The reported cases for R416W show a wide range in disease severity and age of onset, suggesting that epigenetic and/or environmental factors could play key roles in disease progression . In vitro assembly studies showed that the mutation altered filament assembly, inducing the formation of short filament pieces that tend to laterally associate. Transient transfection studies revealed that the level of GFAP expression and the availability of a coassembly-competent endogenous network could influence the phenotypes observed, from the collapse of the whole IF network to the complete integration of the mutant protein. Doxycycline-inducible astrocyte cell lines expressing mutant and WT GFAP were established and confirmed that the R416W GFAP could incorporate into existing GFAP networks, without causing their collapse or altering their distribution. Only when these cells were exposed to oxidative or osmotic stresses were the detrimental effects of the R416W GFAP mutation apparent. These data suggest that this particular disease causing mutation requires either trauma or another stress to precipitate the disease, offering an explanation for the phenotypic variation for this mutation. Our data also establish that filament network disruption is not a necessary prerequisit to identifying the potential of disease causing mutations in IFs, but rather identify mutations where epigenetic factors are likely to have most influence on the appearance and progression of the disease.

317

**Steering Human Vimentin Unit-length Filament (ULF) Formation and Filament Elongation by Systematically Mutating its Coil 1A**

T. Wedig,[1] G. Padilla,[2] P. Burkhard,[2] R. Zimbelmann,[3] U. Aebi,[4] H. Herrmann[1]; [1]Molecular Genetics, German Cancer Research Center, Heidelberg, Germany, [2]The Institute of Materials Science, University of Connecticut, Storrs, CT, [3]Cell Biology, German Cancer Research Center,

Heidelberg, Germany, [4]M.E.Mueller Institute, University of Basel, Biozentrum, Basel, Switzerland

Intermediate filaments (IFs) of the vimentin/desmin-type associate massively from soluble tetrameric complexes into approximately 60 nm long full-width filaments, so-called ULFs, within one second after assembly has been started. We have recently shown that mutation Lys139 to Cys, the first amino acid of linker L1 of human vimentin, stalls assembly at the ULF-stage when carried out at 22°C, whereas at 37°C assembly proceeds normal (Mucke et al., 2004, JMB 340, 97-114). An even more drastic effect is observed upon mutation of Arg113 to Cys in the IF consensus motif NDR in coil 1A. This mutation causes the association of tetramers into distinct, approximately 30 nm-diameter globular particles resembling disordered ULFs (for the corresponding desmin mutation, see Haubold et al., 2003, Cell Motil Cytoskel 54, 105-121). In order to investigate if and how the strength of the inter-helical coiled-coil forming forces - i.e. as assessed by measuring the melting temperature of the corresponding peptides - is correlated with the filament assembly abilities of vimentin, we have systematically mutated a and d positions in coil 1A into hydrophobic amino acids and/or cysteines. Moreover, the cysteine mutants were further analyzed after being cross-linked in and renatured from 4 M urea. Interestingly, while the thus clipped dimers readily assemble into long, unaggregated filaments, their finer ultrastructure and filament width render them different from *bona fide* IFs. Last but not least, the assembly behavior and morphology of these various mutated proteins in vimentin-free cells is presented too.

318

**Mechanical Manipulation of Single IFs in vitro - Towards Understanding the Mechanical Role of IFs in vivo**

L. Kreplak,[1] N. Mücke,[2] H. Bär,[3] J. Leterrier,[4] H. Herrmann,[5] U. Aebi[1]; [1]M.E. Müller Institute, Biozentrum, University of Basel, Basel, Switzerland, [2]Division Biophysics of Macromolecules, German Cancer Research Center (DKFZ), Heidelberg, Germany, [3]Medizinische Universitätsklinik Heidelberg, Heidelberg, Germany, [4]Institut de physiologie et biologie cellulaires, Poitiers University, Poitiers, France, [5]Department of Molecular Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany

Intermediate filaments (IFs) fulfil basic mechanical function in cells and tissues. However, it is still ill defined how keratins, desmin or lamin "function" at the molecular level, respectively. Because of the discovery of dramatic phenotypes in patients presenting point mutations in genes coding for IFs protein such as desmin and keratins, a rational understanding of the mechanical function of IFs is highly needed. We will present findings concerning the mechanics of single IFs in vitro using a scanning force microscopy approach (SFM). The extensibility and toughness of IFs was studied by an online nano-manipulation approach. The SFM tip was used to move or stretch single IFs directly on the support to which they were adsorbed. By this method, IFs were stretched several fold and clear morphological changes were observed, especially a large reduction of the apparent diameter. These results are in good agreement with previous studies on the mechanical properties of macroscopic IF containing fibres such as Hagfish slime threads (Fudge et al. Biophysical Journal (2003), 85, 2015-2027). All in all, the available data support a mechanical shock absorber role for IFs in vivo.

319

**Imaging Native Intermediate Filaments Deposited on Different Solid Supports by Atomic Force Microscopy - Comparison With Simulated Images**

N. Mücke,[1] K. Klenin,[1] R. Kirmse,[1] L. Kreplak,[2] T. Wedig,[3] J. Langowski,[1] U. Aebi,[2] H. Herrmann[3]; [1]Biophysics of Macromolecules, German Cancer Research Center, Heidelberg, Germany, [2]Biozentrum, University of Basel, Basel, Switzerland, [3]Division of Cell Biology, German Cancer Research Center, Heidelberg, Germany

We have imaged by atomic force microscopy (AFM) intermediate filaments (IFs) and IF assembly intermediates after deposition onto different solid supports. AFM of the immobilized IFs and IF intermediates was performed in physiological buffer solution without drying, fixing or washing the samples. As expected, the morphology and biomechanical properties of the adsorbed IFs strongly correlated with the electrostatic properties of the support employed, such as, for example, freshly cleaved mica or hydrophilic glass (cf. Mücke et al. (2004) *J. Mol. Biol*., **335**: 1241-1250). In order to determine the biomechanical parameters of the filaments - for example, the persistence length as a measure of their flexural rigidity - from such AFM images, one has to take into consideration the mechanism governing the deposition processes that, in turn, dictates the filament-surface interactions. To more rationally understand this "capture" mechanism, we performed Brownian dynamics simulations of the stepwise adsorption of 3D polymer chains onto a solid support. Ultimately, these simulations which were animated in a time-lapse series, enabled us to mechanistically interpret the different steps of the filament deposition process as was observed by AFM.

320

**Characterization of Two Different Types of Human Desmin Filaments by Atomic Force Microscopy**

N. Mücke,[1] T. Wedig,[2] J. Langowski,[1] U. Aebi,[3] H. Herrmann[2]; [1]Biophysics of Macromolecules, German Cancer Research Center, Heidelberg, Germany, [2]Division of Cell Biologie, German Cancer Research Center, Heidelberg, Germany, [3]Biozentrum, University of Basel, Basel, Switzerland

We have investigated the properties of filaments assembled from human desmin under different ionic strength conditions (2mM sodium phosphate, pH 7.5, 20-100 mM KCl). Desmin filaments grew much faster than vimentin filaments in 100 mM KCl where huge networks formed within minutes. In 20 mM KCl, desmin assembly was much slower and network formation was suppressed. By atomic force microscopy (AFM) desmin filaments formed in 20 mM KCl exhibited morphological and biomechanical properties that were distinct from those obtained in 100 mM KCl. More specifically, in 20 mM KCl the filaments exhibited a height of 6 nm compared to 9 nm in 100 mM KCl, most likely indicating a decrease in the number of dimers per filament cross-section. Second, a significant decrease in the flexural rigidity of the mature desmin filaments was measured at the lower ionic strength. Third, the filaments exhibited a tendency to form 'closed rings' after several hours of incubation in 20 mM KCl, indicating that end-to-end fusion of the filaments had occurred. Last but not least, we document that the 6-nm high filaments that formed in 20 mM KCl were able to fuse end-on with thick filaments polymerized in 100 mM KCl.

321

**Mechanisms of Cardioprotection by Desmin Cytoskeleton: Targeting Permeability Transition Pores at Mitochondrial Contact Sites**

Y. Capetanaki,[1] K. Rapti,[1] E. Soumaka,[1] M. Mavroidis,[1] N. Weisleder[2]; [1]Cell Biology Division, Center of Basic Research, Foundation of

Biomedical Research, Academy of Athens, Athens, Greece, [2]Cell Biology, Baylor College of Medicine, Houston, TX
We have demonstrated that desmin, the muscle specific intermediate filament (IF) protein, is very important in muscle maintenance and its absence leads to muscle degeneration with extensive fibrosis and calcification, cardiomyopathy and heart failure. Human mutations in desmin or other cytoskeletal proteins, directly or indirectly associated to desmin network, are linked to development of similar defects and heart failure. Thus, it is impoprtant to understand the mechanisms by which disturbance in this cytoskeletal network leads to the onset and development of cardiomyocyte death. Our studies have demonstrated that the most prominent and earliest detected feature of the desmin null phenotype is defects in mitochondrial shape, number, integrity and function. We try to understand the mechanisms leading to these defects and the ways by which these mitochondrial abnormalities lead to cell death. Towards this goal, complementation genetics, atlas arrays and proteomic analyses were used and demonstrated that indeed mitochondrial abnormalities must be the major target and primary cause of the observed cardiomyopathy, most possibly as a consequence of alterations in the mitochondrial contact sites due to the absence of desmin. More specifically, among others, changes in proteins important for the permeability transition pore function, such as porin (VDAC) and its associated proteins hexokinase, creatine kinase and small heat shock proteins, have strongly suggested that this channel might be an important target. Indeed, our first success in ameliorating mitochondrial abnormalities, cardiomyocyte death, cardiomyopathy and heart failure, by overexpressing bcl2, an antiapoptotic protein located at the mitochondrial contact sites, known to regulate VDAC function, strongly supports our hypothesis. It further suggests that IFs might participate either in the correct targeting of proteins to these sites or their stabilization there thus potentially controlling mitochondrial membrane permeability and cell survival.

322

## Modeling Intermediate Filament Networks

S. Portet; Mathematics, University of Manitoba, Winnipeg, MB, Canada

Mathematical models can be helpful in the study of intermediate filament assembly and organization. We have proposed a model to study the dependence of the structural organization of these networks on the mechanical extracellular environment. The model phenomenologically describes the mechanical environment and its effects on the cell. The environment and the elastic properties of filaments jointly determine the structural organization of networks. Computational simulations provide three-dimensional architectures of the network for particular mechanical environments. As this model is very complex, we are developing simplified versions in order to be able to study the dynamics of the different states of intermediate filament material.

323

## Effect of Alexander Disease-causing Mutations on GFAP Filament Assembly and Network Organisation

M. Perng, S. Wen, T. Gibbon, R. Quinlan; School of Biological Sciences, University of Durham, Durham, United Kingdom

Alexander disease (AD) is a rare, but often fatal neurological disorder characterised by the abundant presence in astrocyte of protein aggregates containing the astrocytic intermediate filament (IF) protein glial fibrillary acidic protein (GFAP). Recently, de novo missense mutations in the GFAP have been reported to be associated with nearly all forms of this disease. In this study, we investigate the biological effects of several GFAP mutations on the in vitro assembly and intracellular distribution of IF networks in a range of cell lines with different IF expression profiles. These results were compared with those obtained previously for other GFAP mutations. GFAP mutations located within the rod domain had relatively minor effects on filament assembly and network formation. In vitro, GFAP with internal mutations are able to self-assemble into typical IFs. Although these GFAP mutants failed to form normal filament networks in IF-free cells, they incorporated into filamentous networks in cells expressing co-assembly partners, such as vimentin and GFAP. The most striking effects on IF assembly and network formation were observed with mutations at the highly conserved motifs, namely the LNDR and TYRKLEGGE motifs, at the start and the end of the central rod domain. These mutations perturb both filament assembly in vitro and network configuration in vivo. Similar effects were observed in mutations within the C-terminal tail domain, which affects both filament assembly in vitro and network formation when transiently expressed in cells. These data indicate that individual mutations distributed throughout the GFAP are not equal with the respect to their roles in filament assembly and network formation. These results have important implications for the wild variability in the age of onset and phenotypic severity of the AD.

323A

## KIF5A is Required for Both Anterograde and Retrograde Movement of Neurofilaments in Axons

A. Uchida, A. Brown; Center for Molecular Neurobiology, The Ohio State University, Columbus, OH

Neurofilaments are transported bidirectionally along axons in a rapid infrequent manner. The retrograde motor is dynein but the anterograde motor is unknown. Recently the Goldstein lab reported that targeted deletion of KIF5A in mice causes neurofilament accumulation in DRG cell bodies and a reduction in axonal caliber with no apparent effect on organelle transport, suggesting that KIF5A is an anterograde neurofilament motor. To test this hypothesis, we cultured neurons from wild type (WT) and KIF5A-/- mice. Both sympathetic (SCG) and sensory (DRG) KIF5A-/- neurons extended axons that contained neurofilaments. To examine neurofilament transport, we performed time-lapse fluorescence imaging on SCG neurons expressing GFP-tagged neurofilament protein. The frequency of both anterograde and retrograde neurofilament movement was reduced significantly compared to WT ($p<0.001$, K-S test; average reduction=75%). For those neurofilaments that moved, the average anterograde velocity (excluding pauses) was reduced significantly ($p=0.001$, K-S test; average reduction=52%) but the average retrograde velocity was not ($p=0.4$, K-S test). Immunostaining revealed apparently normal dynein/dynactin levels in KIF5A-/- axons and Western blotting revealed normal levels of dynein/dynactin and kinesin light chains in KIF5A-/- brain. Expression of full-length KIF5A in KIF5A-/- SCG neurons rescued the frequency of neurofilament movement in both directions and partially rescued the anterograde velocity. Expression of a “dominant negative” headless KIF5A in WT SCG neurons completely inhibited movement in both directions. Collectively these data indicate that (1) KIF5A is the principal but not exclusive anterograde motor for neurofilaments in SCG neurons, (2) proteins with homology to KIF5A, probably KIF5B and/or KIF5C, may also be capable of moving neurofilaments anterogradely, and (3) KIF5A is necessary for normal retrograde movement of neurofilaments, which suggests a direct or indirect interaction between the dynein and kinesin motors.

## Focal Adhesions (324-341)

324

### Focal Adhesion Protein and p130Cas Coordinately Regulate *Salmonella typhimurium* Invasion

J. Shi, J. E. Casanova; Cell Biology, University of Virginia, Charlottesville, VA

Cellular invasion by *Salmonella typhimurium*, a gram-negative bacterial pathogen colonizing intestinal epithelia, involves recruitment of focal adhesion proteins to the sites of infection. However, whether these focal adhesion proteins play a role in *Salmonella* entry remains largely undefined. Here, we demonstrate that focal adhesion kinase (FAK) and p130Cas localize to the sites of bacterial infection despite the fact that integrins are not engaged by *Salmonella.* Both FAK and Cas are required for *Salmonella* uptake as cells genetically deficient in either protein (FAK-/- or Cas-/-) exhibit a marked reduction in bacterial colonization, and reconstitution of these cells with wild-type proteins restores bacterial internalization. Surprisingly, bacterial internalization does not require FAK kinase activity, but does require its ability to interact with Cas. In agreement with this finding, *Salmonella* infection stimulates the formation of a complex containing FAK, Cas and paxillin. Moreover, infection of Cas-/- cells leads to formation of enlarged phagocytic structures containing aberrantly organized actin, suggesting that Cas is necessary for proper formation of the phagocytic apparatus. Together, these findings reveal a novel role for focal adhesion proteins in the invasion of host cells by *Salmonella.*

325

### Focal Adhesion Kinase and Src Phosphorylations in HGF-Induced Proliferation and Invasion of Human Cholangiocarcinoma Cell Line, HuCCA-1

V. Leardkamolkarn,[1] U. Pongchairerk,[1] J. Guan[2]; [1]Anatomy, Mahidol University, Bangkok, Thailand, [2]Molecular Medicine, Cornell University, Ithaca, NY

**Abstract** **AIM:** To study the role of FAK (focal adhesion kinase) and its association with Src in HGF induced cell signaling in cholangiocarcinoma progression. **METHODS:** Previously isolated HuCCA-1 cells were re-characterized by immuno-staining and RT-PCR assay for the expressions of cytokeratin19, HGF and c-met mRNA. Cultured HuCCA-1 cells were treated with HGF and determined for cell proliferation and invasion effects by MTT and invasion assays. Western blotting, Immunoprecipitation and Co-immunoprecipitation were also performed to study the phosphorylation and interaction of FAK and Src. A novel Src inhibitor (AZM555130) was applied in cultures to investigate the effects on FAK phosphorylation inhibition and on cell proliferation and invasion. **RESULTS:** HGF enhanced HuCCA-1 cell proliferation and invasion by mediate FAK and Src phosphorylations. FAK-Src interaction occurred in a time dependent manner where Src was proved to be an upstream signaling molecule to FAK. The inhibitor to Src decreased FAK phosphorylation level in correlation with the reduction of cell proliferation and invasion. **CONCLUSION:** FAK plays a significant role in signaling pathway of HGF responsive cell line derived from cholangiocarcinoma. Autophosphorylated Src, induced by HGF, mediates Src kinase activation which subsequently phosphorylates its substrate, FAK, and signals to cell proliferation and invasion. **Keywords:** Human cholangiocarcinoma; Hepatocyte growth factor; C-met; Focal adhesion kinase; Src

326

### Focal Adhesion Kinase Modulates Cell Adhesion Strengthening

K. E. Michael,[1] N. D. Gallant,[2] S. K. Hanks,[3] A. J. Garcia[1,2]; [1]Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, [2]Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, [3]Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN

Integrin-mediated cell adhesion to extracellular matrices (ECM) plays critical roles in numerous physiological and pathological processes. Matrix-bound integrins associate with the cytoskeleton and cluster to form focal adhesions (FA), which link the cytoskeleton to ECM and trigger signaling pathways. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase with multiple targets that is essential to development and regulates FA turnover, cell spreading, and migration. We analyzed the contributions of FAK to adhesion strengthening using FAK-/- cells engineered to re-express WTFAK and FAK mutants, directed at the Y397-autophosphorylation and Y576/577-catalytic sites, under a tetracycline-inducible promoter and a hydrodynamic adhesion strength assay. Micropatterned substrates were used to provide controlled adhesive areas and isolate FA assembly from gross changes in cell shape. Adhesion strength over time was characterized as a logarithmic rise to maximum with parameters of steady-state adhesion strength and strengthening rate (inverse time constant). Compared to FAK-/- cells, WTFAK re-expression significantly reduced steady-state adhesion strength (29%) and increased strengthening rate (5-fold) whereas expression of Y397F-FAK decreased strengthening rate (52%) and Y576/577F-FAK revealed no differences from FAK-/- cells. Quantification of the number of bound alpha5 or alphaV integrins on fibronectin surfaces at steady-state revealed no differences for all cell types, demonstrating that the differences in steady-state adhesion strength were not due to differences in integrin binding. This modulation in adhesion strength can be explained by differences in the localization and distribution of FA in the adhesive area. Immunofluorescence staining at steady-state showed less FA-containing area occupied by vinculin for cells expressing WTFAK (44%); however, no differences were detected for talin. No differences in steady-state vinculin localization were observed for cells expressing Y397F-FAK or Y576/577-FAK. These results indicate that FAK is directing composition and position of FA molecules, preferentially vinculin turnover, thereby reducing steady-state adhesion strength.

327

### Supervillin Modulates Cell Adhesion through a Binding Site for the Focal Adhesion Protein, TRIP6/ZRP-1

N. Takizawa,[1] T. C. Smith,[1] T. Nebl,[1] J. L. Crowley,[1] L. M. Lifshitz,[2] L. M. Hoffman,[3] M. C. Beckerle,[3] E. J. Luna[1]; [1]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA, [2]Department of Physiology, University of Massachusetts Medical School, Worcester, MA, [3]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Cell-substrate adhesion is regulated at focal adhesions (FA) on the plasma membrane. We report here that supervillin (SV)-a peripheral membrane protein that also binds myosin II, F-actin, and calponin-inversely regulates cell adhesion, vinculin-associated FA, and stress fiber formation. In COS-7 cells, a 90% RNAi-mediated knockdown of SV increased by 180% cell adherence to fibronectin in a centrifugation assay (300 x g, 5 min). A FA regulatory sequence within SV amino acids 342-571 (SV342-571) dramatically decreased FA and stress fibers when expressed in COS-7

cells. SV342-571 also decreased the ability of CV-1 cells to deform flexible substrates, a measure of FA and stress fiber function. Potential FA-associated targets were identified by yeast two hybrid analysis and tested for associations with SV and SV fragments in immunofluorescence co-localizations and GST protein pull-downs. We found specific interactions between SV342-571 and two of the three members of the zyxin protein family: SV342-571 binds to the LIM domains of thyroid receptor-interacting protein 6 (TRIP6)/zyxin-related protein 1 (ZRP-1) and lipoma-preferred protein (LPP), but not to the LIM domains of zyxin itself. SV and full-length TRIP6 co-localized when overexpressed in COS-7 cells, and endogenous SV and TRIP6 co-localized within the large FA of A7r5 smooth muscle cells. SV knockdown reduced co-localization of TRIP6, but not zyxin, with vinculin at FA. Overexpression of TRIP6 LIM domains, but not zyxin LIM domains, partially rescued the disruptive effects of SV, SV1-830 and SV342-571 on stress fiber formation. Full-length TRIP6 partially reversed the stress fiber-disrupting effect of full-length SV and SV1-830, but not that of SV342-571. Our results suggest that SV342-571 may disrupt FA by recruiting TRIP6 to FA and that additional SV sequences may modulate TRIP6 function. Supported by NIH grants GM33048 and GM50877.

328

**CaMK-II Activation Promotes Detachment from the Extracellular Matrix**

C. A. Easley,[1] M. O. Faison,[2] R. M. Tombes[3]; [1]Biochemistry, Virginia Commonwealth University School of Medicine, Richmond, VA, [2]Biology, Virginia State University, Petersburg, VA, [3]Biology, Virginia Commonwealth University, Richmond, VA

Fibronectin, a multiadhesive glycoprotein, enables cell adhesion to the extracellular matrix through the β1 subunit of integrin receptors in NIH 3T3 cells. Through interaction with its receptor, fibronectin is known to induce a localized increase in cytosolic $Ca^{2+}$ levels. In this study we first sought to determine whether CaMK-II was the target of the $Ca^{2+}$ flux upon fibronectin binding to β1 integrin. Here, we show that plating cells on fibronectin increases CaMK-II activity in a dose-dependent manner. We also show that disruption of the integrin β1-fibronectin interaction via mechanical rotation, the CD29 integrin β1 antibody, and RGD peptide (GRGDNP) significantly reduces CaMK-II activity. These data suggest that CaMK-II is a target of the $Ca^{2+}$ flux induced by the β1 integrin-fibronectin interaction. We show that ectopically-expressed GFP-linked CaMK-II is enriched at the leading and trailing edges of motile cells. Transfection with constitutively active CaMK-II induces a rounded morphology of cells when subsequently plated on fibronectin. Also, we show that an inhibition of CaMK-II by KN-93 and the myristoylated autoinhibitory peptide (mAIP) causes cells to attach to fibronectin-coated dishes more rapidly than untreated cells, but these treated cells show no dynamic motility after adhering. Taken together, these data suggest that upon attachment of cells to fibronectin through β1 integrin, a $Ca^{2+}$ flux activates CaMK-II, and activated CaMK-II promotes detachment from the extracellular matrix at the leading and trailing edges of NIH 3T3 cells.

329

**Rho-small GTPase Regulates Cell Adhesion Strengthening via Modulation of Focal Adhesions and FAK**

H. Shin,[1] H. Radhakrishna,[2] A. J. Garcia[1]; [1]Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, [2]Biology, Georgia Institute of Technology, Atlanta, GA

Cell adhesion represents a coordinated process of integrin binding to extracellular matrix components, recruitment of cytosolic adhesive molecules, and formation of focal adhesions. The dynamic remodeling of focal adhesions correlates with many cellular processes such as cell cytokinesis, differentiation, and motility. Although it is well established that members of the Rho family of small GTPase play central roles in cytoskeletal assembly and architecture, little is known about how Rho GTPase regulates the adhesion strengthening processes. In this study, we have tested the hypothesis that Rho GTPase modulates cell adhesion strengthening via reorganization of focal adhesions. We have previously demonstrated that focal adhesion composition is regulated by actin-myosin contractility, thereby modulating cell adhesion strengthening. To test the effect of Rho GTPase in cell adhesion, we measured the adhesion strength of NIH3T3 fibroblasts subjected to Rho activation via lysophosphatidic acid (LPA). The focal adhesion size and position were controlled by plating cells on fibronectin-coated surfaces micropatterned via micro-contact printing of self-assembled monolayers of alkanethiols. Treatment of fibroblasts with LPA increased cell adhesion strength in a dose dependent manner. At the highest concentration of LPA, cell adhesion strength increased 45% relative to serum-starved conditions. Immunofluorescence staining for vinculin and talin revealed that LPA stimulate assembly of focal adhesion within the micropatterned substrate. These results suggest that Rho GTPase activation by LPA is involved in regulation of cell adhesion strengthening through focal adhesion assembly.

330

**Rac1 Activation and Translocation to Matrix Adhesion Sites Is Facilitated by FAK**

F. Chang, C. Lemmon, D. Horvitz, L. Romer; Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD

FAK, a cytoplasmic protein tyrosine kinase, is activated and localized to focal adhesions upon cell attachment to extracellular matrix. The observations that FAK null cells spread poorly, and exhibit different focal adhesion arrays suggest that FAK may be involved in focal adhesion turnover and the promotion of cell spreading. Rac1 is a member of the Rho-family GTPases that promotes extension of the leading edge, membrane ruffling, and cell spreading. We investigated Rac1 activation and subcellular location in FAK null and FAK re-expressing MEF. FAK re-expressers had a more robust pattern of Rac1 activation following cell adhesion to fibronectin than the FAK null cells. Translocation of Rac1 to the tips of focal adhesions was observed in FAK re-expressers, but rarely in FAK null cells. Experiments with constitutively active Rac61L and dominant negative Rac17N demonstrated that the activation state of Rac1 determines its localization to focal adhesions in either the presence or the absence of FAK. βPIX is a Rac/Cdc42-specific GEF that activates Rac1. Our results showed that like Rac1, βPIX was also recruited to focal adhesion sites upon attachment to fibronectin and may be responsible for the activation of Rac1 and its recruitment to focal adhesion sites. FAK appears to have a role in Rac1 activation and focal adhesion translocation that may be mediated by βPIX.

331

**Visualizing Dynamic Structures in Migrating F9-Derived Parietal Endoderm**

K. A. LaMonica,[1] T. Hong,[1] J. Mulrooney,[2] L. B. Grabel[1]; [1]Department of Biology, Wesleyan University, Middletown, CT, [2]Department of Biomolecular Sciences, Central Connecticut State University, New Britain, CT

Visualizing Dynamic Structures in Migrating F9-Derived Parietal Endoderm Control of cell migration is essential for proper development.

Unregulated migration can lead to tumor cell metastasis. F9 teratocarcinoma cells form embryoid bodies consisting of an inner core of undifferentiated stem cells surrounded by an outer layer of visceral endoderm. When embryoid bodies are plated on laminin substrates, parietal endoderm migrates away from the embryoid body as a sheet of cells, and this outgrowth is reminiscent of parietal endoderm migration in vivo. Parietal endoderm outgrowth contains leading edge structures such as lamellipodia and filopodia, and adhesion structures such as focal adhesions and focal complexes. Our previous data has shown that perturbation of the Rho signaling pathway at the level of its downstream effector ROCK and over-expression of p190 Rho GAP lead to increased parietal endoderm migration and formation of lamellipodia, and a decrease in focal adhesions. On the other hand, inhibition of the ERK signaling pathway, at the level of MEK and Raf, leads to decreased migration and an abundance of stable focal adhesions. These data suggest that Rho provides the brakes and ERK provides the wheels during parietal endoderm migration. Using live cell imaging techniques, under control conditions, we observe abundant filopodia and lamellipodia formation in the outer ring cells of the outgrowth. Behind the outer edge, cells migrate as a sheet, maintaining close association with each other. We are now observing how migratory and adhesion structures are altered when ROCK or ERK are inhibited. We are also investigating the roles of Rac in directing lamellipodia extension at the leading edge of the outgrowth.

332

**Differential Phosphorylation of Cortactin Regulates Lamellipod Extension Through Alterations in Adhesion Plaque Dynamics**

A. E. Kruchten, E. W. Krueger, M. A. McNiven; Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN

Cortactin is an actin binding protein upregulated in several cancers and a substrate of the tyrosine kinase Src. ERK and Src phosphorylation of cortactin has been implicated in positive and negative effects on actin polymerization *in vitro*, respectively. Surprisingly, Src phosphorylation of cortactin has been shown to promote actin dependent cell migration and enhance the metastatic capacity of cells *in vivo*. The GOAL OF THIS STUDY was to directly test the roles of cortactin phosphorylation on actin assembly and motility in living fibroblasts and metastatic human pancreatic cells. Several different phospho-forms of cortactin altered at key tyrosines or serines were expressed and changes in lamellipod extension, focal adhesions, and actin assembly quantified. Expression of a phospho-mutant of cortactin mimicking Src phosphorylation (Y384/429/445E) resulted in a dramatic extension of lamellipodia in the absence of stimulation, while the phospho-blocking mutant (Y384/429/445F) inhibited lamellipodia formation. Because dendritic actin polymerization supports membrane extension and migration, we next examined this process *in vivo* using an “actin comet” formation assay. A cortactin mutant mimicking ERK-mediated serine phosphorylation (S368/381D) resulted in a two-fold increase in comet formation while the tyrosine mutants had no effect To test how tyrosine phosphorylation promotes lamellipodial extension while inhibiting actin polymerization, the effects of cortactin mutants on focal adhesions and cell spreading were examined. Interestingly, the cells expressing cortactin Y384/429/445E exhibited faster spreading on fibronectin resulting in larger cells with more numerous focal adhesions and decreased cortactin association. From these observations, we predict that tyrosine phosphorylation plays a role in the turnover of focal adhesions and subsequent cell motility and metastasis while serine phosphorylation promotes actin polymerization. These findings provide some of the first insights into a role for differential cortactin regulation of focal contact function and cell adhesion.

333

**Focal Adhesion Kinase Couples Lpa Signaling to Focal Adhesion Retrograde Flow and Subsequent Changes in Cell Shape**

M. Iwanicki, T. Vomastek, R. Tilghman, K. Martin, J. T. Parsons; Microbiology, University of Virginia, Charlottesville, VA

The protein tyrosine kinase, Focal Adhesion Kinase (FAK) is an essential molecule in mediating beta1-dependent integrin signaling. Upon integrin engagement FAK becomes activated as evidenced by phosphorylation of tyrosine 397. FAK also becomes activated in response to growth factors such as Lysophosphatidic Acid (LPA), Thrombospondin, Bombasin and Epidermal Growth Factor. Activation of FAK initiates downstream signaling that has been implicated in regulation of cell migration and survival. We have utilized siRNAs for FAK to attenuate FAK levels in Rat-2 fibroblasts and have examined the role of FAK in LPA induced cell shape and changes in focal adhesion dynamics. Rat -2 fibroblasts with reduced FAK expression become elongated, exhibiting long axon like extensions. These extensions are rich in polymerized actin and microtubules. Attenuation of FAK expression in Rat-2 fibroblasts results in decreased responsiveness to LPA stimulation manifested in the inhibition of activation of the ERK pathway and PI3 kinase pathways. In addition, LPA stimulation of control siRNA cells but not FAK siRNA cells resulted in changes of ERM proteins phosphorylation suggesting that FAK is important in ERM mediated cell shape changes (*Lamb R.F , Current Biology 1997, 7: 682-688*). Inhibition of ROCK but not MEK, PI3K or MLCK in Rat 2 fibroblasts resulted in the characteristic elongated cell morphology, suggesting that ROCK is a component of FAK signaling. Finally, LPA stimulation of control siRNA but not FAK siRNA Rat 2 cells resulted in focal adhesion retrograde flow. Treatment of control siRNA Rat 2 cells with ROCK inhibitor abrogated retrograde flow of focal adhesions. These data strongly point to a model in which FAK couples LPA/ROCK signaling to the dynamic regulation of focal adhesions and subsequent changes in cell shape.

334

**Elucidating the Role of Myosin II Contraction in Adhesion Protein Dynamics and Maturation**

A. S. Kirby,[1,2] Y. Zilberman,[1,3] D. B. Weibel,[1,4] L. C. Kapitein,[1,5] C. Waterman-Storer[1,6]; [1]Physiology Course 2005, Marine Biological Laboratory, Woods Hole, MA, [2]Cell Biology, Duke University, Durham, NC, [3]Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel, [4]Chemistry and Chemical Biology, Harvard University, Cambridge, MA, [5]Physics and Astronomy Laser Center, Vrije University, Amsterdam, The Netherlands, [6]Cell Biology, Scripps Research Institute, La Jolla, CA

Cells move by coupling forces generated in the actin cytoskeleton to the extracellular matrix via trans-membrane focal adhesions. RhoGTPase-regulated myosinII contraction of the cytoskeleton linked to adhesion induces integrin clustering and focal adhesion “maturation.” We hypothesize that tension-induced maturation of focal adhesions results in changes in protein composition and dynamics that may influence adhesion morphology, strength, and signaling. We first aimed to elucidate the relationship between contractility, maturation and protein binding/dissociation at focal adhesions. We performed FRAP of GFP-conjugated integinα5, FAK, paxillin, zyxin, talin, and vinculin expressed in mouse embryo fibroblasts. Protein dynamics fell into two categories, those with low fluorescence-recovery halftimes (t1/2) (<25s; talin, paxillin FAK and zyxin), and those more stably bound in adhesion sites (integrinα5, t1/2~100s; vinculin, t1/2~250s). Cells were treated with myosinII inhibitors to reduce contractility or transfected with activated Rac1 to induce immature focal complexes, and FRAP was performed on integrinα5, talin, and vinculin.

Talin and integrin dynamics were relatively insensitive to these treatments, while t1/2 of GFP-vinculin was greatly reduced by both activated Rac1 (~80s) and myosinII inhibition (<50s). We next sought to determine how induction of myosinII contraction promotes organization of actin bundles and maturation of adhesions. Cells co-expressing cherry-actin and GFP-paxillin were treated with myosin inhibitors to disassemble focal adhesions. Contraction was induced by drug washout and cells were analyzed by time-lapse fluorescence microscopy. During contraction, no de-novo adhesion formation was seen, rather focal complexes matured into focal adhesions by fusion of small preexisting clusters that increased GFP-paxillin fluorescence over time. Nascent actin bundles appeared to grow from fusing focal complexes, as opposed to being recruited from preexisting f-actin. These results indicate that myosinII contraction promotes maturation of adhesions by induction of actin filament growth and tight vinculin binding.

335

**Dissect the Relationship among of Focal Adhesions, Traction Forces, and Extracellular Matrix Organization**

W. Guo, Y. Wang; Physiology, UMASS Medical School, Worcester, MA

Migration of adherent cells involves the assembly-disassembly of focal adhesions, the exertion of traction forces, and the organization of the extracellular matrix (ECM). While previous studies have demonstrated a close relationship between the formation of nascent focal adhesions and the generation of strong traction forces at the leading edge, the function of traction forces remains unclear. In addition, it is puzzling that strong traction forces disappear underneath most focal adhesions as they mature, without concurrent movement or dissociation of focal adhesions from the ECM. To address these questions, we have plated 3T3 fibroblasts on flexible polyacrylamide substrates embedded with fluorescent microbeads and coated with fluorescent collagen or fibronectin. In addition, cells were transfected with GFP proteins to label focal adhesions. Simultaneous imaging of beads, focal adhesions, and the ECM allowed us to determine the relationship among substrate deformation, ECM organization, and focal adhesion assembly and disassembly. Our results suggest that the exertion of traction forces is coupled to the movement and organization of the underlying ECM. Therefore energy associated with traction forces may be used for the organization of ECM structures, which may in turn coordinate the migration of a population of cells during tissue formation and wound repair.

336

**The Role of Git1 Tyrosine Phosphorylation in Cell Spreading**

E. J. Kelly, J. E. Casanova; Dept. of Cell Biology, University of Virginia, Charlottesville, VA

Git1 is a GAP for ARF family GTPases that localizes to focal adhesions, the plasma membrane and cytosolic sites. In addition to its role as an Arf GAP, GIT1 also serves as a scaffolding protein, interacting with the Rac-GEF Pix through a central domain and the focal adhesion component paxillin through its C-terminus. The Git1/PIX complex mediates delivery of the p21 activated kinase, Pak to focal adhesions and the plasma membrane where it regulates both actin cytoskeleton and focal adhesion dynamics. Git1 becomes phosphorylated on multiple tyrosines in response to integrin ligation, but the role of these modifications in Git1 function is not known. We have identified four tyrosine residues (Y293, Y392, Y519 and Y607) whose mutation to phenylalanine alters the level of GIT1 phosphorylation. Mutation of Y392, Y519 or Y607 reduces the overall GIT1 phosphotyrosine level under normal growth conditions. Intriguingly, mutation of the fourth site, Y293, not only does not diminish phosphorylation, it actually leads to increased overall phosphorylation. All four mutants localize normally to focal adhesions, indicating that phosphorylation does not regulate focal adhesion targeting. To test the role of these phosphorylation events in cell behavior we examined their effects in a spreading assay in which cells were plated onto fibronectin. Surprisingly, we found that neither wild-type nor a catalytically inactive mutant of GIT1 impairs spreading, suggesting that GAP activity is not important in this process. Similarly, mutation of either Y392, Y519 or Y607 has no effect. In contrast, mutation of Y293 significantly slows spreading. Interestingly, Y293 lies within the binding site for PIX and we are currently working to determine if phosphorylation regulates the interaction with PIX.

337

**Erk1/2-Dependent Caldesmon Phosphorylation Regulates Podosome Turnover in A7r5 Smooth Muscle Cells**

Z. Gu, C. Hai; Molecular Pharmacology, Physiology & Biotechnology, Brown University, Providence, RI

Podosomes are focal adhesion-like cytoskeletal structures associated with the release of metalloproteases and degradation of extracellular matrix during cell invasion. PKC activation by phorbol dibutyrate (PDBu) has been shown to remodel actin stress fibers into F-actin-enriched podosome columns in A7r5 vascular smooth muscle cells, but the underlying signaling mechanism is not fully understood. Caldesmon is an actin-binding protein that stabilizes actin filaments against the action of actin-severing proteins in vitro. Furthermore, the MEK/Erk/Caldesmon phosphorylation cascade is an important mechanism of PKC-mediated contraction of smooth muscle cells. In this study, we tested the hypothesis that MEK1/2 and Erk1/2-dependent caldesmon phosphorylation regulates PKC-mediated podosome formation in A7r5 smooth muscle cells. We found that PDBu stimulated the phosphorylation of Erk1/2 and caldesmon by 15- and 20-fold, respectively. Immunofluorescence microscopy with Z-sectioning of PDBu-stimulated cells indicated colocalization of phospho-MEK1/2, phospho-Erk1/2, phospho-caldesmon and caldesmon at the ring of podosome columns. The MEK1/2 inhibitor, U0126 completely abolished PDBu-stimulated Erk and caldesmon phosphorylation. U0126-treated cells were still able to form podosomes associated with diminished levels of phospho-MEK1/2, phospho-Erk1/2 and phospho-caldesmon. However, the rate of podosome formation was significantly attenuated in U0126-treated cells. Overexpression of caldesmon by 180% (relative to β-actin) using caldesmon-GFP transfection induced the formation of podosome with caldesmon enriched in the core. Live-imaging of caldesmon-GFP dynamics during PDBu-induced podosome formation in A7r5 cells indicated a long lag time before the formation of podosomes and a slower turnover rate of podosomes. siRNA-knockdown of caldesmon expression by 70% induced the loss of some central stress fibers in A7r5 cells, however podosomes could still form in response to PDBu treatment. These results suggest that caldesmon is a regulator of podosome turnover rather than an essential structural component of podosomes in A7r5 smooth muscle cells.

338

**Tes is a Negative Regulator of Mena**

B. Boeda,[1] D. Briggs,[2] B. Garvalov,[1] N. McDonald,[2] M. Way[1]; [1]Cell Motility Laboratory, Cancer Research UK, London, United Kingdom, [2]Structural Biology Laboratory, Cancer Research UK, London, United Kingdom

It is now widely accepted that Ena/VASP family members are key players in regulating actin filament assembly during cell migration in a variety of different organisms. Studies from many groups have shown that the cellular localization of Ena/VASP family members is dependent on the association of their EVH1 domains with F/LPPPP motifs in binding partners such as zyxin, vinculin or lamellipodin. We now report that Tes, a LIM domain-containing protein, that is localised to focal adhesions interacts directly with the EVH1 domain of Mena. Surprisingly, this binding does not involve the canonical "FPPPP" sequence, but the LIM3 domain of Tes. We have solved the structure of the LIM3:EVH1 complex to obtain insights into this novel interaction. The crystal structure reveals that the LIM3 domain of Tes occludes the FPPPP binding site of the EVH1 domain of Mena. This suggests that the LIM3 domain of Tes may modulate the binding between the EVH1 domain of Mena and its FPPPP motif-containing partners. We present a variety of in vivo and in vitro evidence to support this hypothesis. In addition, the affinity of the Tes LIM3 domain for the EVH1 domain of VASP was drastically lower than that for Mena, suggesting that Tes may specifically regulate Mena but not VASP.

339

**Dominant-negative Alpha-actinin Disrupts Focal Adhesion Organization and Sensitizes Osteoblasts to Tumor Necrosis Factor-alpha-induced Apoptosis**

J. W. Triplett, F. M. Pavalko; Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN

Maintenance of bone structural integrity is thought to depend greatly on the rate of apoptosis of bone-forming osteoblasts. Substrate adhesion is thought to be an important regulator of apoptosis, since a lack of adhesion results in anoikis. Focal adhesions are sites of cell attachment to, and signaling between, the substrate in osteoblasts. We hypothesize that α-actinin is positioned to play an important role in both focal adhesion organization and signaling, since it can interact with both structural and signaling components within focal adhesions. To test this, we over-expressed a dominant-negative form of α-actinin (ROD-GFP) that displaces endogenous α-actinin from focal adhesions, thus preventing the recruitment of actin to focal adhesions. Immunofluorescence and morphometric analysis of focal adhesions in MC3T3-E1 osteoblasts revealed that ROD-GFP expression caused a 40% reduction in focal adhesion area and long-axis length. To investigate the role of α-actinin in apoptosis, UMR106 osteosarcoma cells expressing ROD-GFP or a control GFP were treated with tumor necrosis factor-α (TNF-α), in the presence of cycloheximide, for a period of four hours. Preliminary experiments indicate that ROD-GFP expression alone does not induce apoptosis, but sensitizes cells to the apoptotic effects of TNF-α. The apoptotic markers histone 2A.X phosphorylation, caspase-3 cleavage, and poly-ADP-ribose polymerase cleavage were significantly increased in ROD-GFP expressing cells treated with TNF-α compared to GFP-expressing controls. To investigate the mechanism underlying this phenomenon, we assessed the ability of TNF-α to induce the known survival signal of nuclear factor-kappa B (NF-κB) nuclear translocation. By immunofluorescence, TNF-α-induced NF-κB nuclear translocation was inhibited in cells expressing ROD-GFP compared to controls. These data suggest that α-actinin may normally play a role in TNF-α induced signaling by promoting survival signals through the TNF receptor, either directly or through focal adhesions.

340

**Structure of the Alpha-Actinin-Vinculin Head Domain Complex Determined by Cryo-Electron Microscopy**

K. A. Taylor,[1] D. F. Kelly,[1] D. W. Taylor,[1] C. Bakolitsa,[2] A. Bobkov,[2] R. C. Liddington[2]; [1]Institute of Molecular Biophysics, Florida State University, Tallahassee, FL, [2]Dept. of Cell Adhesion and Extracellular Marix Biology, The Burnham Institute, La Jolla, CA

We have determined the vinculin binding site on α-actinin using cryoelectron microscopy of 2-D arrays formed on phospholipid monolayers doped with a nickel chelating lipid. Chicken smooth muscle α-actinin cocrystallized with HIS-tagged β1-integrin cytoplasmic tail and a vinculin fragment containing residues 1-258 (VD1). VD1 occupied a single site on α-actinin with 60-70% occupancy in a difference 3-D map relative to α-actinin alone. In these arrays, the two ends of α-actinin, each containing an actin binding and calmodulin-like domain, are held in distinctly different environments. The VD1 difference density has a shape consistent with its atomic structure, namely a pair of four helix bundles. The atomic model of the complex juxtaposes the α-actinin binding site on VD1 with the N-terminal lobe of the α-actinin calmodulin-like domain. These results establish this methodology for determining the structure of weakly interacting species in a membrane-like environment. Differential Scanning Calorimetry (DSC) confirms that the interaction between full-length α-actinin and the vinculin head domain (vinculin domains 1-3, VD1-3) is specific but weak. DSC also shows that the interaction between α-actinin and VD1-3 is significantly stronger when α-actinin is truncated at the N-terminus to remove the actin-binding domains. We propose that combinatorial interactions, such as α-actinin binding to the β-1 integrin tail allowing for stronger binding to VD1, regulate these binding events such that they only occur at prescribed sites in the cell such as focal adhesions.
Supported by NIH grant GM64346 to the Cell Migration Consortium.

341

**Generation and Characterization of Talin2 Mutant Mice**

N. Chen, Y. Liao, S. Lo; Center for Tissue Regeneration and Repair, University of California-Davis, Sacramento, CA

Talin is a large focal adhesion component that interacts with multiple molecules, such as integrins, focal adhesion kinase (Fak), vinculin, layilin and actin. A close related member, talin2, has been identified. Although talin2 is highly homologous to talin, many of its potential binding activities and physiological roles remain to be established. Talin1 knockout in mice leads to embryonic lethality due to impaired cell migration at the gastrulation stage. Here, we reported the characterization of mice carrying a beta-galactosidase insertion at talin2 gene, the distribution of talin2 in various tissues, and the identification of a testis splice form of talin2. Using a talin2 gene-trapped embryonic stem cell clone, we have developed a talin2 mutant mouse line that expresses the talin2 fused with beta-galactosidase. Crossing between heterozygous mice produced pups with expected Mendelian distribution of genotype ratio, indicating that this talin2 mutant did not lead to embryonic lethality. Using the RT-PCR and Northern blot analysis, we confirmed the gene-trap insertion has disrupted talin2 transcripts. In the testis, talin2 expresses as a shorter form with a unique 30 residues at N-terminus linking to a common C-terminus of the long form. However, it did not seem to affect the morphology of testis or reproduction of male mice. In fact, male and female mutant mice are fertile. Utilizing the expression of talin2/ beta-galactosidase fusion protein, we have examined the distribution of talin2 in tissues by X-gal staining. In contrast to talin1, talin2 expression is more restricted in tissues and cell types. Interestingly, most of the staining was concentrated on the basement membrane of the cells, suggesting that talin2, like talin1, was localized to the cell-matrix junctions. These data demonstrate the expression patterns of talin2.

## Cell-Cell Adherens Junctions (342-357)

342

### Turnover Dynamics of P120-catenin in Adherens Junctions : A Quantitative Analysis Using Fast 3D Imaging 0f FRAP

P. Kaiser,[1] C. Gueudry,[1] J. Sibarita,[2] A. Bershadsky,[3] F. Amblard[1]; [1]Physics dept, Curie Institute, Paris, France, [2]Biology dept, Curie Institute, Paris, France, [3]Dept Molecular Cell Biology, Weizmann Institute of Sciences, Rehovot, Israel

Adherens junctions (AJs) are complex molecular assemblies, based on cadherin transmembrane receptors, that perform adhesive interactions between contacting cells. AJs are thought to have some degree of plasticity, but their dynamic properties are poorly known. In particular, the molecular turnover rates of AJ components have not been assessed. p120-catenin interacts with the juxta-membranal cytoplasmic site of cadherin, and is required for AJ stability. We studied the turnover of p120-GFP in MCF-7 epithelial cells, using a novel instrument that employs fast 3D imaging to measure fluorescence recovery after laser mediated photobleaching. This enabled us generate a rapid sequence : (i) pre-bleach 2D-3D imaging, (ii) photobleaching user-defined target (spot, lines, polygonal regions) using fast moving mirors, and (iii) immediate imaging of the recovery in 2D-3D mode. The experimental parameters under control are : laser bleaching pulse power and duration, imaging rate. AJ were typically photobleached during 50-100 ms, and recovery was imaged with a 100 ms time resolution. FRAP on AJ depends on local, cytosolic p120 diffusion and on its dissociation rate from AJ. These two kinetic components were resolved by assessing the diffusion coefficient of p120 away from AJs, and then subtracting the computed diffusive component from the AJ recovery. Cytosolic diffusion coefficient was assessed at several points from the recovery of the 2D radial concentration profile. This procedure is robust to image noise and to irregular cell geometry. It yields typical diffusion coefficients of 10 micron^2/s. Recovery at AJs, once corrected for their diffusive component, led to a typical residence time of 10 seconds. Although this time might depend on p120 the expression level, it is much shorter than the 2 minutes time measured for ezrin at the epithelial apex.

343

### The Armadillo Protein Plakophilin 2 is Required for Assembly of the Intermediate Filament-Binding Protein Desmoplakin into Desmosomes

A. E. Bass, L. M. Godsel, E. V. Amargo, K. J. Green; Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL

Plakophilin 2 (Pkp2), a member of the p120-subfamily of armadillo repeat proteins, is localized in the cytoplasmic plaque of desmosomes where it partners with a range of desmosomal proteins, including desmoplakin (DP). While ectopic overexpression of Pkp2 has been shown to enhance DP's recruitment to cell-cell borders, its specific role in desmosome assembly is unknown. Using a combination of fixed and live cell imaging and RNAi-mediated knockdown, we examined Pkp2's role in this process. Within minutes following cell-cell contact, intermediate filament-associated, DP-rich particles appeared in the cytoplasm, and subsequently were translocated to cell-cell borders where they were incorporated into nascent desmosomes. Confocal analysis of cells fixed over time following calcium-induced cell-cell contact revealed that Pkp2, but not desmosomal cadherins or the armadillo proteins plakoglobin or Pkp3, selectively co-distributed with DP particles. DP-GFP-expressing keratinocytes were imaged live and then fixed and stained to identify proteins that co-localize with assembly-competent particles. 100% of DP-GFP particles that coalesced and moved toward cell-cell contacts contained Pkp2, but none contained desmocollin or Pkp3. While some DP-GFP particles contained plakoglobin, translocation was not dependent on its presence. To directly test Pkp2's role in assembly, we used siRNA to reduce Pkp2 to <10% of control levels. Loss of Pkp2 inhibited DP particle formation and localization at cell-cell borders, whereas the distribution of other desmosomal and adherens junction proteins was unaffected. Pkp2 was also observed to co-localize with microfilaments, and actin but not microtubule perturbation impaired DP-precursor trafficking. These results suggest that junctional plaque precursor assembly and accumulation at nascent desmosomes requires Pkp2, and raises the possibility that Pkp2 might facilitate precursor translocation through interactions with the actin-based cytoskeleton.

344

### Eps8 is Necessary for Efficient E-Cadherin-Dependent Cell-Cell Adhesion in Human Epithelial Cells

C. Ballara,[1] G. Scita,[2] A. Disanza,[2] S. Alema[1]; [1]Istituto di Biologia Cellulare, Consiglio Nazionale delle Ricerche, Monterotondo, Italy, [2]IFOM-IEO, Milan, Italy

Epithelial cells adhere to their neighbours through specialized structures such as adherens junctions (AJ), whose core is composed of cadherins. Intercellular adhesion is a dynamic process that involves the protrusion of filopodia that orchestrate clustering of AJ proteins. Eps8, a substrate of receptor tyrosine kinases, in a complex with Abi1 and Sos-1 induces Rac activation leading to actin cytoskeleton remodelling. Recent studies have shown that Eps8 regulates the growth of actin filaments by capping their barbed ends, an activity essential for cell motility. Here, we report that Eps8 localizes at cell-cell junctions in both fibroblasts and epithelial cells. Eps8 is recruited to intercellular contacts once epithelial cell-cell adhesion is initiated and interacts with complexes containing E-cadherin and p120 catenin. We show using RNAi that reduction in Eps8 expression leads to delayed AJ formation as well as defects in cortical actin organization. Furthermore, ablation of Eps8 inhibits HGF-dependent cell scatter. These effects are reversed by reintroduction of Eps8 into RNAi cells. This study provides novel functional information on Eps8 as a cell-cell adhesion protein and increases our understanding of the roles of actin capping proteins in epithelial adhesion.

345

### Glis2: a Novel p120 Catenin-Binding Protein

C. R. Hosking, Y. Fujita; MRC Laboratory for Molecular Cell Biology, University College London, London, United Kingdom

p120 catenin (p120ctn) is an Armadillo family member, which binds to the cytoplasmic tail of cadherins at sites of cell-cell contact. It has been implicated in regulation of cell-cell adhesion and actin dynamics through its association with members of the Rho-GTPase family. However, the presence of p120ctn in the nucleus observed for a number of cell lines indicates an additional nuclear function for this protein. Using the yeast two-hybrid method, our lab has shown that p120ctn can bind to the transcription factor Glis2, a protein homologous to the Gli family that are transducers of Sonic Hedgehog signaling. We also show that p120ctn expression can induce C-terminal cleavage of the Glis2 protein. This cleavage is increased by the overexpression of Src, suggesting a role for Src-mediated tyrosine phosphorylation of p120ctn. The cleaved form of Glis2 retains its ability to bind p120ctn and DNA. Since Glis2 has been shown to have a role in neuronal differentiation, we investigated the effect

of overexpressing Glis2 in the neural tube of developing chick embryos. Interestingly, overexpressing Glis2 inhibited neuronal differentiation. This was also observed when the cleaved form of Glis2 was coexpressed with p120ctn. These results suggest a nuclear role for p120ctn by modulating the cleavage of the transcription factor Glis2, and potentially altering the transcription of genes in the neuronal differentiation pathway.

346

**Role of Rap1 in Regulating Epithelial Cell Junction**

S. Asuri,[1,2] N. C. Paranavitana,[1,2] L. A. Quilliam[1,2]; [1]Biochemistry and Molecular Biology, Indiana University, Indianapolis, IN, [2]Walther Cancer Institute, Indianapolis, IN

The defined architecture and function of epithelial tissues depend on cell-cell adhesion. E-cadherin mediates the primary epithelial cell junctions; adherens junctions (AJ). The goal of our study was to understand the role of the Ras-related GTPase, Rap1, in the dynamics of these E-cadherin based junctions. Individual MDCK epithelial cells showed Rap1 distributed both around the cell membrane and in the cell interior. When cells begin to establish contacts, Rap1 became enriched in this region and localized to cell junctions in established monolayers. Rap1 cycles between active GTP and inactive GDP bound states. We measured Rap1 activity in MDCK epithelial cells during formation and disruption of AJs following "calcium switching". Rap1 was activated within 2 min of calcium-chelation/cell junction disruption and this activity was sustained for at least 30 min. Rap-GTP levels were subsequently reduced upon $Ca^{2+}$ re-addition to the growth medium. Stable expression of a constitutively active Rap1A (Rap63E) mutant promoted dramatic “compaction” of MDCK cells, implying increased cell-cell contact. Whereas, dominant negative (Rap17N) mutant imparted a more “flattened” phenotype. Cells expressing Rap63E resisted hepatocyte growth factor-induced disruption of adherens junctions and cell scattering, while Rap17N cells showed accelerated cell scattering. We next explored the possibility that Rap1 influences AJ by regulating the activity of Rho proteins. Rap63E activated Cdc42 in HEK-293T cells whereas Rap17N expression reduced Cdc42 activation during $Ca^{2+}$-switching in MDCK cells. We also found that Rap1 bound FRG1, a Cdc42 GEF that has been implicated in adherens junction formation (Fukuhara et al JCB 166, 393, 2004). In summary, Rap1 is activated during disruption of junctions, yet is essential for the formation and maintenance of junctions. We therefore hypothesize that Rap1 is activated when cell junctions are disrupted and is thus “charged” to perform its functions during their reformation.

347

**Establishing Epithelial Cell Surface Polarity of AQP3 Upon Cell-cell Adhesion**

L. N. Nejsum, S. Yamada, W. Nelson; Molecular and Cellular Physiology, Stanford University Medical School, Stanford, CA

Aquaporins have distinct polarized localizations within the same cell: for example in collecting duct principal cells, AQP2 is apical and sub-apical, AQP3 is basal-lateral, and AQP4 is mainly basal, but little is known regarding the mechanisms involved in polarized sorting and organization of aquaporins. We sought to define the distribution of AQP3 during the early stages of development of cell polarity and formation of the basal-lateral membrane domain following cadherin mediated cell-cell adhesion. A Madin-Darby Canine Kidney (MDCK) cell line stably expressing AQP3 tagged with pEGFP was generated to follow AQP3 distribution using live cell imaging. In a single non-polarized cell, AQP3 was evenly distributed throughout the plasma membrane both on the open surface and the surface bound to the coverslip. Live cell imaging revealed that during initial cell-cell adhesion AQP3 accumulates precisely within the boundaries of the cell-cell contact and overlaps the distribution of E-cadherin-dsRed. To follow the delivery of AQ3 to the membrane, a synchronized pool of AQP3 tagged with photoactivatible (PA) GFP was photoactivated in the TGN; PAGFP-AQP3 appeared and accumulated at the site of initial cell-cell adhesion. This is the first time a non-adhesion protein has been shown to be targeted to the site of initial cell-cell adhesion following exit from the TGN. These results suggest that mechanisms involved in post-Golgi vesicle trafficking to the developing basal-lateral plasma membrane are spatially established rapidly following initiation of cadherin-mediated cell-cell adhesion.

348

**Role of Formin-1 in Endothelial Cell Adherens Junction Formation**

B. M. Gaudet,[1] S. D. Burchat,[2] J. W. R. Copeland[1]; [1]Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, [2]Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada

Adherens junctions (AJs) form between adjacent cells through the homophilic interaction of transmembrane cadherins. In epithelial cells, following cadherin engagement, the intracellular domain of E-cadherin recruits β-catenin which, in turn, recruits α-catenin. α-catenin indirectly links the AJ to the actin cytoskeleton through its interaction with actin-binding proteins such as vinculin, α-actinin or formin-1. Formin-1, like other formins, contains two formin homology domains, FH1 and FH2. The FH1 domain recruits profilin-bound G-actin to the growing barbed end of an actin filament, while the FH2 domain directly nucleates actin polymerization. Recruitment of formin-1 to the AJ by α-catenin is mediated by a coiled-coil motif located N-terminal to the Formin-1 FH1. Like epithelial cells, endothelial cells also form AJs, but the role of formin-1 in AJ formation in these cells has not been investigated. We have demonstrated by RT-PCR analysis that mRNA transcripts of Formin-1 isoforms I, IV and V are expressed in human dermal microvascular endothelial cells. An antibody to the FH2 domain of murine Formin-1 has been produced and should recognize all Formin-1 isoforms. This antibody also recognizes the Formin-1 homologues from chick and humans. We are investigating the role of Formin-1 at the endothelial cell adherens junction by examining the ability of activating and interfering derivatives of Formin-1 to affect AJ formation induced by the serum mitogen sphingosine-1-phosphate. The results will be assessed by immunofluorescence using our Formin-1 antibody as well as by vascular permeability assays. Furthering our understanding of AJ formation and remodeling in endothelial cells is of great interest given the essential role these structures play in the normal functioning of the endothelium as well as in clinically relevant processes such as tumour-induced angiogenesis.

349

**Co-regulation of Cadherin and Receptor Tyrosine Kinases by Intracellular Trafficking**

S. R. Joseph, D. M. Bryant, J. L. Stow; Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland, Australia

E-cadherin is a classical adhesion protein important for such processes as establishment of polarity, maintenance of epithelial barrier function, and suppression of metastases in epithelial cells. We have previously shown that at steady state E-cadherin undergoes a dynamic trafficking through

endosomes, both during exocytosis and for recycling. Furthermore we have shown that this process can be regulated by receptor tyrosine kinases. Here we examine the intracellular trafficking of E-cadherin with EGF and FGF receptors. EGF and FGF are both able to induce internalisation of E-cadherin, with different fates for internalised protein. Similarly, localization of cognate EGF and FGF receptors is dependent on epithelial polarization or E-cadherin function in epithelial cells, suggestive of conjoint RTK/E-cadherin relationship. Long-term exposure of epithelial cells to growth factors revealed differential induction of morphological changes. Surprisingly, examination of endocytic sorting machinery revealed that growth factors also differ in their routes of E-cadherin and receptor trafficking at early timepoints after stimulation. Accordingly, distribution of E-cadherin and receptors to different endosomal compartments resulted after EGF or FGF stimulation. These findings are important in understanding differences in signalling and for transient versus permanent downregulation of cadherin-mediated adhesion.

350

**Polycystins and Adherens Junction Proteins are Integrated in a Signaling Membrane Domain**

T. Roitbak,[1] Z. Surviladze,[1] R. Tikkanen,[2] R. Sandford,[3] A. Wandinger-Ness[1]; [1]Pathology, University of New Mexico, Albuquerque, NM, [2]Biochemistry II, University of Frankfurt, Frankfurt am Main, Germany, [3]Medical Genetics, University of Cambridge, Cambridge, United Kingdom

Polycystins are plasma membrane proteins that serve to regulate epithelial cell polarity and differentiation through their function as part of several multiprotein complex with epithelial adhesive junction proteins. Defects in polycystin protein function are causal in autosomal dominant polycystic kidney disease (ADPKD) and thought to precipitate changes in cell polarity and differentiation. Biochemical and morphological studies have been used to show that the polycystins and the associated proteins E-cadherin and β-catenin distribute in a complex with the raft marker flotillin-2, but not caveolin-1. The integrity of the polycystin multiprotein complex was sensitive to cholesterol depletion as shown by cyclodextrin treatment of immunoprecipitated complexes. The C-terminus of polycystin-1 is sufficient for flotillin-2 association. Flotillin-2 was found to contain CRAC cholesterol binding domains and to promote plasma membrane cholesterol recruitment. Based on coassociation of signaling molecules, such as Src kinases and LAR protein tyrosine phosphatases, we propose that the polycystin/adherens junction multiprotein complex is embedded in a cholesterol-containing signaling microdomain specified by flotillin-2. The domain is thought to be important in the regulation of stable cell-cell adhesion. Work supported by NIDDK 50141, PKDF 12(A-C)2R, NKF F758 and DFG SFB628.

351

**Loss of *Smad5* in the Gut Epithelium Causes E-Cadherin Mislocalization to the Basal Membrane and Disregulation of Migration**

J. F. Schmouth,[1] B. A. Auclair,[1] A. Zwijsen,[2] D. L. Gumucio,[3] N. Perreault[1]; [1]Anatomy and Cell Biology, University of Sherbrooke, Fleurimont, PQ, Canada, [2]Developmental Biology, Catholic University of Leuven, Leuven, Belgium, [3]Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

**BACKGROUND**: Bone morphogenic proteins (BMPs) are multipurpose proteins associated with the TGF-beta superfamily. The BMP proteins interacting with their receptor will trigger a signalisation cascade that is driven by the SMADs transcription factors (Smad 1, 5 and 8). Interestingly, *Smad5* null mice die at mid-gestation due to multiple embryonic defects including a vestigial gut. This suggests that *Smad5* may play an essential role in the intestine morphogenesis and cellular function. **AIM:** To investigate the in vivo function of *Smad5* in intestinal organogenesis and in intestinal epithelial cellular process such as proliferation, differentiation and migration. **METHODS AND RESULTS:** With the use of the Cre/loxP system, we generated a mice with *Smad5* exclusively deleted in the gut epithelium by crossing floxed *Smad5* mice with the Villin-Cre line. Histological analysis revealed an elongation of the cryptal compartment and an important lengthening of the villi in the *Villin-Cre/Smad5*$^{loxP/loxP}$ mice. Incorporation of BrdU during 90 minutes revealed no modifications in the number or localization of the proliferating cells in mutant mice. However, incorporation of BrdU during 48 hour made it possible to highlight an increased speed in the migration of the epithelial cells along crypt/villus axis in the mutant animals. Immunostaining with an E-cadherin specific antibody showed a change of localization in the mutant mice. E-cadherin is normally found at *Zonula Adherens* establish in the lateral membrane of intestinal epithelial cells. In the floxed *Smad5* animals, we observed a relocalization of E-cadherin mainly to the basal membrane. Western blot analysis showed an increase in protein expression of E-cadherin in the mutant mice. **CONCLUSION:** These results suggest that *Smad5* may play an important role in intestinal epithelial migration by affecting E-cadherin localization.

352

**The Wilm's Tumor-1(WT1) Interacting Protein is Expressed in the Glomerular Podocyte and Regulates Assembly of the Cell-Cell Contacts**

A. Mukherjee,[1] J. Kim,[2] A. Padiyar,[1] J. Romanello,[1] S. Schechtman,[1] M. Koniezkowski,[1] J. R. Schelling,[1] J. R. Sedor[1]; [1]Medicine/Nephrology, Case Western Reserve University, Cleveland, OH, [2]Physiology, Case Western Reserve University, Cleveland, OH

Podocyte differentiation is critical for glomerular filtration barrier function and regulated by WT1, a zinc finger transcription factor. We identified a novel, WT1 interacting protein (WTIP) that maps to human chromosome 19q13.1, a region with familial focal segmental glomerulosclerosis genes. Our hypothesis proposes WTIP normally monitors slit diaphragm and glomerular basement membrane assembly and shuttles into the nucleus after podocyte injury, providing a link between altered slit diaphragm structure and gene expression.To define WTIP function, we generated recombinant adenovirus which ectopically expressed GFP-WTIP fusion protein and found to target to cell-cell contacts. GFP-WTIP colocalized with ZO-1, beta-catenin and pan-cadherin in podocyte cell junctions. WTIP was also found to be present at ends of F-actin filaments in cultured podocytes. Similar to ZO-1 and beta-catenin, WTIP targeting to cell-cell junctions was calcium-dependent suggesting its association with adherens junctions. In subcellular fractionation experiments with rat glomeruli, WTIP is found to be co-fractionated with nephrin and caveolin. This suggests its association with nephrin in podocyte lipid rafts which comprise the slit-diaphragm in vivo. Our data indicate that WTIP is important for assembly and maintenance of adherens type cell junctions in podocytes and that of slit-diaphragm in vivo.

353

**Neuronal Cell Adhesion Molecule L1 Regulates Motility and Adherens Junction Formation in Breast Cancer Cells**

M. Shtutman, E. Levina, M. Baig, P. Ohouo, I. Roninson; Cancer Center, Ordway Research Institute, Albany, NY

Regulation of adherens junction formation and cell motility is important for tissue remodeling and tumor metastasis. Neuronal cell adhesion molecule L1 (L1-CAM) was recently found to play a role in growth regulation of breast cancer cells (1). To understand the function of L1-CAM in tumor cells, we studied the effects of L1-CAM knockdown and overexpression in MCF-7 breast carcinoma cell line. We find that L1-CAM knockdown enhances adherens junction formation and decreases cell motility, while elevation of L1-CAM leads to disruption of E-cadherin based cell-cell adhesion and enhancement of cell motility. In dense cultures with strengthened adherens junctions, expression of L1CAM is down-regulated; there is evidence that this down-regulation may occur through inhibition of β-catenin signaling (2). Overexpression of a truncated L1-CAM lacking the cytoplasmic domain has no effect on cell motility, indicating that intact cytoplasmic domain is required for proper functioning of L1-CAM. Our results suggest a new mechanism for tumor invasion through elevation of L1-CAM expression in tumor cells, leading to local disruption of adherens junctions and cell migration toward the tumor edge, where further elevation of L1 expression is followed by acceleration of tumor invasion and dissemination. 1.Primiano et al, Cancer Cell 2003 2.Gavert et al, J. Cell Biol, 2005

354

**Analysis of δ-Catenin Mutations and Modifications in Cancer**

Y. Chen, B. Jeansonne, S. K. Bareiss, Q. Lu; Department of Anatomy and Cell Biology, The Brody School of Medicine at East Carolina University, Greenville, NC

δ-Catenin, or NPRAP/neurojungin, is a unique armadillo domain-containing protein in that it is primarily expressed in the central nervous system. However, recent analysis of the human genome revealed consistently an association of *δ-catenin* mRNA sequences with cells of cancerous origin. In our most recent studies, Western blot and Tissue Micro-Array immunohistochemistry revealed a close association of increased δ-catenin expression with human primary prostatic adenocarcinomas (Lu et al., Human Pathology, 2005). δ-Catenin expression increased with prognostically significant increased Gleason scores, which was accompanied by the down regulation and redistribution of E-cadherin and $p120^{ctn}$, major cell-cell junction proteins whose inactivation is often linked to the aggressive phenotype of prostate cancer. These studies indicate that δ-catenin may be directly involved in prostate cancer progression. Presumably, the δ-catenin dependent activities increased in cancer either by the presence of dominant mutations or by an accumulation of δ-catenin protein available for pathological interactions. We therefore first compared *δ-catenin* cDNA sequences obtained from prostate cancer and benign tissue samples by RT-PCR method. No mutations were identified in the entire coding region of *δ-catenin*. This was also true for *δ-catenin* cDNA isolated from the esophageal cancer tissue samples, indicating that δ-catenin mutations in primary sequences were less likely the prominent feature in cancer. Interestingly, biochemical analyses showed that overexpressed δ-catenin underwent significant posttranslational modifications that included its phosphorylation, proteolytic processing, and redistribution in response to growth factor stimulation. In addition, overexpressed δ-catenin interacts with actin cytoskeleton and alters the cellular morphology. These data favor a model in which overexpressed δ-catenin accumulates and redistributes abnormally in cancer cells, leading to an increased interaction with cancer specific signaling pathways. This study was supported by American Cancer Society and the Department of Defense.

355

**Different Functions for Different Coxsackievirus and Adenovirus Receptor Isoforms**

K. J. Excoffon,[1] M. R. Avenarius,[2] N. D. Gansemer,[1] G. L. Traver,[1] R. J. Smith,[2] J. Zabner[1]; [1]Internal Medicine, University of Iowa, Iowa City, IA, [2]Otolaryngology, University of Iowa Hospitals and Clinics, Iowa City, IA

The Coxsackievirus and Adenovirus Receptor (CAR) has several significant biological functions including mediating viral infection and cell:cell adhesion. The gene encoding human CAR, *Cxadr*, is thought to consist of 7 alternatively spliced exons. Mouse *Cxadr* consists of 8 alternatively spliced exons with 2 possible c-termini: one that is similar to human *Cxadr* terminating in exon 7, the other terminates in exon 8. The last four amino acids of both isoforms encode a similar PDZ binding domain. We hypothesized that a human exon 8 form of CAR also exists. A genomic screen for human exon 8 revealed the mouse exon 8 ortholog. RT-PCR on human cell lines and tissues demonstrate expression of either $hCAR^{exon7}$ alone or both $hCAR^{exon7}$ and $hCAR^{exon8}$ combined. Notably, lung and primary cultures of polarized human airway epithelia (HAE) predominantly express $hCAR^{exon7}$. After subcloning the cDNA for $hCAR^{exon8}$, we expressed it in heterologous cell lines and compared it to $hCAR^{exon7}$. Both isoforms demonstrate similar expression levels as determined by Western blot, localization at cell:cell junctions, and adenovirus binding and infection. Interestingly, whereas $hCAR^{exon7}$ co-expression with the PDZ domain interacting protein MAGI-1b alters the localization of MAGI-1b to cell junctions, $hCAR^{exon8}$ does not. We further hypothesized that expression of $hCAR^{exon8}$ would confer HAE with a distinct phenotype. $HCAR^{exon7}$ localizes to the basolateral side of HAE providing an inherent protection from apical viral infection. In contrast, $hCAR^{exon8}$ is apical making HAE permissive for apical viral infection. These results show a differential regulation and localization of these CAR isoforms that may explain the observed differences in viral infection in distinct tissue compartments.

356

**Cyclic Nucleotides in Junction Restructuring During Spermatogenesis: An *In Vivo* Study**

O. Sarkar; Center for Biomedical Reasearch, Population Council, New York, NY

A plethora of evidence is presently available which demonstrates the role of cyclic nucleotides in junction restructuring. For example, numerous studies have shown cAMP and cGMP to be key regulators of junction assembly and disassembly in different *in vitro* and *in vivo* systems. In this study, we examine the role of guanylate cyclase in junction restructuring in the seminiferous epithelium of the rat testis. We demonstrate for the first time the structural interaction of soluble guanylate cyclase (sGC) with tight and adherens junction proteins in the testis. By immunoprecipitation, sGC was found to interact with occludin, JAM-A and ZO-1, as well as with cadherin, catenin, nectin, afadin, ponsin and espin, illustrating its role in cell junction dynamics. These results were corroborated by immunohistochemistry experiments which showed sGC to localize at the site of the ectoplasmic specialization. Furthermore, administration of Adjudin (formerly known as 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide), a chemical entity known to specifically perturb adhesion between Sertoli cells and germ cells (primarily round and elongate spermatids with some spermatocytes) resulted in a ~2-fold increase in sGC, coinciding with the loss of germ cells from the seminiferous epithelium. More importantly, the ability of sGC to associate with occludin during Adjudin-mediated junction restructuring in the testis was completely lost (note: the integrity of the tight junction, which constitutes the blood-testis barrier, was not affected by Adjudin treatment), whereas its association with cadherin was elevated. Taken collectively, these results clearly demonstrate that sGC function is restricted to the cadherin-

catenin complex to mediate the loss of Sertoli-germ cell contact.

357

**Laminin-333/α6β1 Integrin is a Non-basement Membrane Cell Adhesion Protein Complex at the Ectoplasmic Specialization of the Rat Testis**

H. Yan, C. Cheng; Population Council, New York, NY

Apical ectoplasmic specialization (ES) is a testis-specific Sertoli-germ cell actin-based adherens junction (AJ) type restricted to the interface between Sertoli cells and spermatids. Laminin γ3 was shown to be a putative binding partner of α6β1 integrin at the apical ES. However, the α and β chains that can constitute a functional laminin receptor for α6β1 integrin at the apical ES are unknown. Using RT-PCR and immunoblottings to survey all laminin α, β and γ chains in cells of the seminiferous epithelium, it was noted that α2, α3, β1, β2, β3 and γ3 chains were found in germ cells, whereas α1, α2, β1, β2, γ1 and γ3 were in Sertoli cells, implicating α3 and β3 are the plausible laminin chains restricted to germ cells that can be the bona fide partners of γ3. To verify this postulate, different cDNA constructs based on the Domain I of α3, β3 and γ3 were subcloned into pET101/D-TOPO® vector. Recombinant proteins were expressed in *E. coli* and purified using Ni-columns. Monospecific polyclonal antibodies against the α3, β3 and γ3 chains were raised in rabbits. Studies by immunoblottings, immunohistochemistry and immunofluorescence microscopy using testes and Sertoli/germ cell cocultures demonstrated that laminin α3, β3 and γ3 chains of 165, 140, 146 kDa, respectively, were indeed restricted to germ cells at the apical ES and co-localized with each other and β1 integrin. The protein levels of these three laminin chains and β1 integrin were also induced when functional apical ES was established in Sertoli-germ cell cocultures. More important, using co-immunoprecipitation technique, an anti-laminin γ3 antibody also pulled out α3 and β3 and vice versa. In summary, laminin-333 is the functional non-basement membrane receptor of α6β1 integrin at the apical ES.

## Extracellular Matrix & Cell Signaling I (358-378)

358

**Effect of EGF on Redistribution of P65 Nf-*KB*/rela in A431 Cells Spread on Immobilized Ligands**

O. Petukhova,[1] A. Emelyanov,[1] A. Bolshakova,[1] L. Turoverova,[1] V. Babakov,[1] I. Kropacheva,[1] K. I. Magnusson,[2] G. Pinaev[1]; [1]Cell Cultures, Institute of Cytology, St. Petersburg, Russian Federation, [2]Medical Microbiology, Linkoping University, Linkoping, Sweden

The interaction of a cell with its extracellular matrix defines a number of cellular functions, and influences gene expression. The actin cytoskeleton, by engaging membrane receptors, takes part in cell signaling stimulated by adhesive substrates. To study the influence of specific adhesion on transcription factor NF-kB activity, we have analyzed the redistribution of p65 subunits of NF-kB in A431 cells spread on immobilized ligands (fibronectin, laminin 2/4 or antibodies to EGF receptor) under the EGF action. By using double immunofluorescent staining and confocal microscopy, we compared the localization of p65 and actin cytoskeleton organization in the presence and absence of cytochalasin D, known to prevent actin polymerization. We demonstrate that spreading of A431 cells on immobilized ligands was by itself enough to induce partial p65 transport to the nucleus. The redistribution of p65 under the EGF action depended on the particular immobilized ligand, and part of the p65 population colocalized with actin structures. Cytochalasin D treatment of spread cells induced actin cytoskeleton disorganization, and both common and specific features of actin aggregate patterns were found for each immobilized ligand. P65 subunits were observed in the nucleus and cytoplasm. In the latter case, p65 was seen as large and small cytoplasmic aggregates, partly colocalizing with actin aggregates. EGF treatment resulted in reorganization and enlargement of small actin aggregates and accumulation p65 in these regions. We suggest that activated p65 uses the actin cytoskeleton as a matrix for its translocation between the cytoplasm and nucleus, and that binding of p65 to the cytoskeleton occurs in the actin polymerization points. Acknowledgements: The work was supported by RFBR grant 03-04-48251 and the Swedish Institute Visby program.

359

**PDGFRβ-Mediated Phosphorylation of MUC1 Cytoplasmic Tail Promotes Its Nuclear Localization and Enhances Invasiveness in Pancreatic Ductal Adenocarcinoma Cells**

P. K. Singh,[1] R. L. Cerny,[2] M. A. Hollingsworth[1]; [1]Eppley Institute for Research in Cancer and Allied Diseases, UNMC, Omaha, NE, [2]UNL, Lincoln, NE

MUC1 is a heterodimeric transmembrane glycoprotein that is overexpressed and aberrantly glycosylated in ductal adenocarinomas. Differential phosphorylation of the MUC1 cytoplasmic tail (MUC1CT) has been associated with signaling events that contribute to proliferation and metastasis of cancer cells. We directly evaluated the *in vivo* phosphorylation status of MUC1 in pancreatic cancer cell lines by mass spectrometry-based sequence analysis of the MUC1CT. Nanospray LC-MS/MS analysis revealed a novel tyrosine phosphorylation site at the HGRYVPP sequence in MUC1CT, the first report of phosphorylation of tyrosine at this motif. Platelet-derived growth factor receptor β (PDGFRβ) was identified as a potential kinase for tyrosine at the HGRYVPP motif in the MUC1CT by analysis with the Scansite algorithm. It was confirmed by sequence analysis of the products of *in vitro* kinase assays on a synthetic 66-residue MUC1CT peptide with human recombinant PDGFRβ. Stimulation of the pancreatic cancer cell line S2-013.MUC1F with platelet-derived growth factor-BB (PDGF-BB), a potent stimulator of PDGFRβ, enhanced phosphorylation of MUC1CT. Stimulation of S2-013.MUC1F cells with PDGF-BB also increased nuclear localization of MUC1CT and its association partner, β-catenin. Although PDGF-BB stimulation had no significant effect on cell proliferation rate, it enhanced invasion *in vitro* through matrigel. Microarray analysis of PDGF-stimulated cells revealed upregulation of several metastasis related genes as compared to unstimulated or mock transfected, PDGF-stimulated cells. These results provide compelling evidence for the regulation of invasiveness of pancreatic adenocarcinoma cells by PDGFRβ-mediated phosphorylation of the MUC1CT.

360

**Syndecan-1 Expression in Epithelial Cells is Induced by TGFb through a PKA-dependent Pathway**

K. Hayashida, P. Park; Medicine, Baylor College of Medicine, Houston, TX

Syndecans are a major family of cell surface heparan sulfate proteoglycans (HSPGs) and they modulate a wide variety of biological molecules through their heparan sulfate (HS) moiety. Although all syndecans contain the ligand-binding HS chains, they likely perform specific functions *in*

*vivo* because their temporal and spatial expression patterns are different. However, how syndecan expression is regulated has yet to be clearly defined. In this study, we examined how induced expression of syndecan-1 is regulated in epithelial cells. Our results showed that among several bioactive agents tested, only TGFβ2 and forskolin induced syndecan-1 expression on normal murine mammary gland (NMuMG) epithelial cells. TGFβ1 and TGFβ3 also induced syndecan-1 expression on NMuMG cells and other epithelial cells, such as A549 human lung carcinoma and C127 transformed murine mammary gland cells. Interestingly, TGFβ treatment inhibited proliferation of epithelial cells in response to FGF-2 in an HS-dependent manner, suggesting that the inhibitory effects of TGFβ on epithelial cell proliferation are partly mediated by HS chains of syndecan-1. Epithelial syndecan-1 induction by TGFβ was significantly inhibited by transient expression of a dominant-negative PKA cDNA construct and also by specific inhibitors of PKA, such as KT5720 and myristoylated peptide inhibitor, but not by inhibitors of PKC. Steady-state syndecan-1 mRNA was not increased by TGFβ treatment, indicating that TGFβ induces syndecan-1 expression through PKA in a post-transcriptional manner. TGFβ treatment induced phosphorylation of an invariant serine residue in the cytoplasmic domain of syndecan-1. Further, PKA was co-immunoprecipitated from epithelial cell lysates by an anti-syndecan-1 antibody and PKA phosphorylated the syndecan-1 cytoplasmic domain *in vitro*. These findings uncover a novel regulatory mechanism where TGFβ, PKA, and the cytoplasmic domain of syndecan-1 coordinate to regulate expression of syndecan-1 at the cell surface.

361

**Rapidly Dephosphorylated- and Perinuclear Golgi-Translocated-PKCalpha is Upstream of Calcium and PLD in Lacritin Mitogenic Signaling, Respectively Through NFATC1 and mTOR**

N. Wang,[1] J. Wang,[1] R. Raab,[2] R. McKown,[2] G. W. Laurie[1]; [1]Department of Cell Biology, University of Virginia, Charlottesville, VA, [2]Department of Integrated Science and Technology, James Madison University, Harrisonburg, VA

Lacritin is an apically released mitogen of some exocrine glands with suggested roles in targeted epithelial renewal and secretion as it flows downstream. Lacritin binding of syndecan-1 core protein is a key step in lacritin-dependent mitogenesis, but little is known of its mitogenic signaling pathway. Previous signaling studies revealed that lacritin-dependent calcium mobilization was pertussis toxin- ($G\alpha_i/G\alpha_o$), U73122- (phospholipase C) and Go 6976- (protein kinase C) sensitive. Neither p42 nor p44 are activated indicating that lacritin signaling is distinct from the Ras-GTP-dependent pathway utilized by many growth factors. Here we focused on the putative role of PKC in lacritin-responsive HSG cells, and identified downstream effectors. Depletion of PKCα by siRNAs D7 and D10 abrogated lacritin-stimulated (10 nM) mitogenesis without affecting proliferation by FBS. Lacritin and N-24 deletion mutant, but not C-25 deletion mutant, promoted PKCα translocation to the perinuclear Gogli region coincident with PKCα dephosphorylation. Dephosphorylation was detectable by 1 min, returning to baseline by 30 min. PLD1 is also located in the perinuclear Golgi region and is activated by PKCα in response to lacritin. A mitogenic pathway from PLD through mTOR is indicated by the capacity of rapamycin to completely inhibit lacritin-dependent proliferation. Depletion of PKCα inhibited calcium mobilization and NFATC1 nuclear translocation. Inhibition of NFATC1 translocation by cyclosporin also completely inhibited lacritin-dependent mitogenesis. Lacritin's C-terminal binding of syndecan-1 thus leads to targeting of downstream mTOR and NFATC1 via $G\alpha_i$ or $G\alpha_o$/PKCα-PLC/PLD/mTOR and $G\alpha_i$ or $G\alpha_o$/PKCα-Ca/calcineurin/NFATC1 for mitogenesis.

362

**Syndecan-1 as a Coreceptor For Prosecretory Mitogen 'Lacritin' in a Core Protein-Specific and GAG-Free Selective Manner**

P. Ma,[1] S. Beck,[1] R. Raab,[2] R. McKown,[2] G. Coffman,[2] A. Rapraeger,[3] G. W. Laurie[1]; [1]Department of Cell Biology, University of Virginia, Charlottesville, VA, [2]James Madison University, Harrisonburg, VA, [3]University of Wisconsin-Madison, Madison, WI

Lacritin is a small glycoprotein secreted by human lacrimal acinar cells. Lacritin acts in an autocrine or paracrine manner at low nanomolar levels to respectively promote further lacrimal secretion and ductal proliferation. How lacritin targets cells is unknown. Here we report on lacritin affinity purification of several cell surface proteins and identification of syndecan-1 as a putative lacritin co-receptor. The first of approximately four bands repeatedly eluted from lacritin affinity column was determined by sequencing to be syndecan-1. In pull-down assays, lacritin bound mammalian syndecan-1, but not syndecan-2 or 4. Binding is to the core protein, preferentially to a form lacking heparan sulfate glycosaminoglycan. The suggestion that local heparanase activity might regulate lacritin binding to the ubiquitously expressed syndecan-1 was confirmed by RNA interference and mitogenesis. Deletion of lacritin's C-terminal 25 but not N-terminal 5,10, or 24 amino acids eliminated binding. Soluble lacritin and N-terminal 24 deletion mutant but not heparin and C-terminal deletion mutants inhibited binding in competitive binding assays. Syndecan-1 was detected in 'near apical' and basolateral plasma membranes in human lacrimal gland. Syndecan-1, a coreceptor for FGF's, Wnt's and other growth factors appears to serve in the same capacity for lacritin, but in a unique manner apparently independent of heparan sulfate side chains. Such selectivity, apparently heparanase-dependent suggests a dynamic new mechanism by which epithelial renewal can be regulated. Dependence on lacritin's C-terminal 25 amino acids for binding, as well as mitogenesis and calcium signaling, suggests involvement of lacritin's C-terminal amphipathic α-helix in lacritin-receptor interactions. Supported by EY13143 (GWL)

363

**Analysis of the Mammary Epithelial Cell Secretome: Regulation Through Epidermal Growth Factor Receptor Transactivation**

J. M. Jacobs, R. D. Quesenbery, L. E. Kathmann, D. G. Camp, R. D. Smith, B. D. Thrall; Biological Sciences Division, Pacific Northwest National Lab, Richland, WA

The survival, proliferation, and motility of human mammary epithelial cells (HMECs) are thought to depend on proteins secreted or shed into the extracellular space, yet the diversity of proteins released by these cells and the mechanisms underlying their regulation are poorly understood. Here, we report a comprehensive analysis of the "secretome" from an HMEC line. We applied the accurate mass and time tag approach to selectively identify and quantitate via comparative mass spectrometry (MS) proteins released from HMECs both constitutively and in response to the protein kinase C agonist, PMA. In addition to stimulating PKC, PMA causes transactivation of the epidermal growth factor receptor (EGFR), an autocrine pathway required for HMEC growth and motility. Thus, we also investigated the role of EGFR transactivation in regulating protein release from HMECs. We used capillary liquid chromatography (LC) combined with tandem MS to identify peptides and create a mass and time tag database for application in high-throughput LC-Fourier transform-ion cyclotron resonance MS analyses. Nearly 900 proteins were identified confidently from HMEC-conditioned medium, which was consistent with previous predictions of the large diversity of the mammalian secretome. Proteins

whose release was stimulated by PMA treatment included ligands for receptor signaling pathways as well as a variety of proteins with proteolytic function. Selective blockage of EGFR signaling using either an EGFR kinase inhibitor or neutralizing receptor antibody inhibits secretion of a subset of PMA-stimulated extracellular proteins, including several matrix metalloproteinases (MMPs). Regulation of MMP secretion through EGFR transactivation is mediated through transcriptional induction and involves EGFR-dependent activation of the extracellular signal-regulated kinases. Furthermore, cytokine-induced secretion of MMP was also EGFR-dependent, suggesting that EGFR transactivation is a general regulatory mechanism cells use to selectively modify their extracellular environment in a context-dependent manner.

364

**Participation of CTGF in Skeletal Muscle Fibrosis**

C. Vial, P. Cañón, E. Brandan; Cell & Mol. Biol., Catholic University of Chile, Santiago, Chile

Duchenne muscular dystrophy (DMD) is characterized by degeneration of muscle fibres, which leads to progressive muscular atrophy. Regeneration of these is dependent on the activation and proliferation of progenitor myoblasts, process that in DMD is insufficient to compensate for the continuous loss of myofibers and fibrotic scarring ensues. Connective tissue growth factor (CTGF) has been related to fibrotic processes for his ability of inducing connective tissue synthesis. CTGF is augmented in fibrotic disorders, but there is little information regarding its role in skeletal muscle disease, as well as its biological receptor. It has been reported that CTGF binds to the low-density lipoprotein receptor-related protein (LRP), and that it binds directly to integrins. The aim of our study is to elucidate CTGF's role in skeletal muscle biology with relation to pathological fibrosis, and to study its receptor in myoblasts. Here we report that TGF-β and lysophosphatidic acid (LPA), two molecules involved in tissue scaring, induce CTGF expression in C2C12 mouse myoblast cell line in a dose dependent manner. In order to study the effect of CTGF in myoblasts we have generated a recombinant active CTGF (rCTGF). Here we report that incubation of myoblasts with rCTGF stimulates the production of fibronectin, collagen Iα2, and proteoglycans. In order to study CTGF´s receptor we have also use a siRNA to inhibit LRP and RGDS to inhibit integrins, resulting in an altered response to the cytokine. These results suggest that LRP might be an internalization receptor for CTGF and that in DMD not only are fibroblasts involved in the increase of connective tissue, but myoblasts can contribute to muscle fibrosis. (Supported by MDA3790, FONDAP, MIFAB)

365

**A Novel Regulatory Mechanism of TGFβ Signalling by Decorin and LDL Receptor-Related Protein (LRP)**

C. Cabello-Verrugio, M. Paz Marzolo, E. Brandan; Cell & Mol. Biol., Catholic University of Chile, Santiago, Chile

Skeletal muscle differentiation is a process strongly inhibited by TGFβ *in vitro*. Three proteoglycans, whose expression is regulated during myogenesis, are able to bind TGFβ and regulate its biological effects: decorin, biglycan and betaglycan. Decorin, a soluble proteoglycan, can interact with diverse proteins, among them growth factors, extracellular matrix proteins, and cell surface receptors. We have previously demonstrated that myoblast decorin null clone (Dcn null) shows an accelerated differentiation due to decreased sensitivity to TGFβ. Moreover, we have recently demonstrated that LDL receptor-related protein (LRP) is an endocytic receptor for decorin in myoblasts. The aim of this work is to understand the mechanism responsible for the decreased sensitivity to TGFβ in Dcn null myoblasts. We show that Dcn null myoblasts are less responsive to TGFβ than wild type myoblasts, evaluated by TGFβ-dependent p3TPlux reporter activity. This effect is specific for decorin since decorin re-expression in Dcn null myoblasts restores TGFβ's response to wild type levels. This difference in TGFβ's response is not produced by changes in levels of TGFβ receptors, Smad proteins or in TGFβ-dependent Smad-2 phosphorylation. Moreover, we show that in wild type myoblasts RAP (an inhibitor of LRP-ligand endocytosis) or a specific siRNA to LRP both inhibit p3TPlux activity to the level found in the Dcn null myoblasts. The specificity of this effect is demonstrated by re-expressing decorin in Dcn null myoblasts treated with RAP or siRNA to LRP both decrease p3TP-lux activity. Therefore, we demonstrate that decorin regulates TGFβ signalling in myoblasts through a novel mechanism involving LRP but not Smad-2 phosphorylation. (Supported by FONDAP, MIFAB, HHMI, CONICYT AT-24050108)

366

**Cystatin Inhibits TGF-β Mediated Signaling in B16F10 Melanoma**

V. N. Onkoba; Biochemistry, Kirksville College of Osteopathic Medicine, Kirksville, MO

Cysteine proteases are extracellular matrix-degrading enzymes that have been shown to play a critical role in tumor invasion and metastasis of several human cancers. Urokinase plasminogen activator (uPA) and its inhibitor PAI (plasminogen activator inhibitor)-1, both members of the plasminogen activating system, are increased in tumors. Studies have shown that overexpression of PAI-1, as noted in several cancers, increases the invasive capacity of tumor cells thus establishing a pro-metastatic role for PAI-1. Our lab previously showed that cystatin C, a natural cysteine protease inhibitor, plays a role in decreasing invasion in B16F10 melanoma metastasis. Although a previous study by Sokol and Schiemann (2004) showed that cystatin C binds to the transforming growth factor-β1 (TGFβ) type-I receptor, the mechanism by which cystatin C inhibits metastasis still remains unclear. In this study, we focused on downstream effects of the Smad pathway, which is activated following TGF-β1 phosphorylation. We compared uPA, PAI-1, Smad 2/3 and p-Smad 2/3 levels in TGF-β1-treated cells incubated with or without cystatin S. Our methods included: RT-PCR, Western blot and immunofluorescence. Immunofluorescence studies showed that TGF-β1 induced perinuclear and nuclear translocation of p-Smad 2/3. TGF-β1 caused a decrease in uPA message levels whereas p-Smad 2/3 levels increased and Smad 2/3 levels remained unchanged. Western blot analyses showed cystatin S inhibits increased p-Smad 2/3 levels. PAI-1 message levels were also inhibited by cystatin S treatment. Taken together, our findings suggest that cystatins decrease the invasive potential of melanoma via the TGF-β1 signaling pathway and could offer promise as anti-metastatic agents.

367

**Ionizing Radiation Predisposes Human Mammary Epithelial Cells to TGFß induced Epithelial to Mesenchymal Transition**

K. L. Andarawewa, A. C. Erickson, W. S. Chou, M. Barcellos-Hoff; Cancer Biology, Lawrence Berkeley National Laboratory, Berkeley, CA

Ionizing radiation (IR) is a known human breast carcinogen. Many aspects of the association between radiation and breast cancer have been elucidated in the past decades. Previous studies have demonstrated that radiation elicits a rapid and dynamic program of extracellular matrix remodeling that is in part mediated by the activation of TGFß. Although the mutagenic capacity of IR is widely acknowledged as the basis for its

action as a carcinogen, we and others have shown that IR can also induce growth factors and extracellular matrix remodeling. In recent studies we have shown that IR, compromises human mammary epithelial cell (HMEC) polarity and multicellular organization in a manner characteristic of neoplastic progression through a heritable, non-mutational mechanism. Since colonies arising from irradiated HMECs exhibited traits of cancer progression we postulated that IR predisposes HMECs to undergo persistent phenotypic changes and this phenotype is augmented by TGFß. To test this hypothesis we characterized the irradiated phenotype in nonmalignant HMEC in presence or absence TGFß. We have demonstrated that irradiated single HMEC in presence of TGFß can generate a persistent phenotype in daughter cells characterized by change in cell morphology, increased expression of mesenchymal markers, such as N-cadherin, fibronectin and vimentin, decreased expression of epithelial markers, E-cadherin and ZO-1, and increased cellular motility when compared to sham controls or following single treatments. These phenotypic changes are hallmarks of an epithelial to mesenchymal transition (EMT) event which is a signature of an aggressive type of tumor in vivo. Thus, we show IR predisposes HMECs to TGFß induced EMT and promote neoplastic progression.

368

**Adhesion to the G3 Domain of Laminin-5 Promotes the Osteogenic Differentiation of Human Mesenchymal Stem Cells through an ERK Dependent Pathway**

R. F. Klees,[1] R. M. Salasznyk,[1] T. R. Scott,[2] A. Boskey,[3] G. E. Plopper[1]; [1]Biology, Rensselaer Polytechnic Polytechnic, Troy, NY, [2]Department of Animal and Veterinary Sciences, Clemson University, Clemson, SC, [3]Biology, Hospital for Special Surgery, New York City, NY

The overall mechanisms governing the osteogenic differentiation of human mesenchymal stem cells (hMSC) are poorly understood, as well as the role of laminins in bone development. We previously reported that laminin-5 induces an osteogenic phenotype in hMSC and does so through an extracellular signal-related kinase (ERK) dependent pathway. We hypothesized that this is a result of integrin-ECM binding, and that it occurs via the known alpha3 G3 integrin binding domain of laminin-5. To test this hypothesis we cultured hMSC on several different globular domains of laminin-5 in the presence and absence of a specific ERK inhibitor, PD98059. hMSC adhered best to G3, and this adhesion maximally activated ERK within 120 minutes. Prolonged culturing (16 days) of hMSC on G3 led to activation and expression of key osteogenic markers (bone sialoprotein 2, osteocalcin, alkaline phosphatase) in hMSC, and induced a significant increase in the phosphorylation of the master bone gene Runx2. These effects were reduced when cells were cultured in the presence of PD98059. G3 binding did not increase matrix mineralization, demonstrating that G3 alone is not sufficient to induce complete osteogenic differentiation in vitro. We conclude that G3 mediates attachment of hMSC to laminin-5 and that this adhesion stimulates most, but not all, of the osteogenic differentiation associated with laminin-5 binding to hMSC.

369

**Mechanical Crosstalk and Feedback Between Integrins and ERK**

M. J. Paszek,[1] N. Zahir,[1] K. R. Johnson,[1] M. Dembo,[2] D. A. Hammer,[1] V. M. Weaver[3]; [1]Bioengineering and Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA, [2]Biomedical Engineering, Boston University, Boston, MA, [3]Pathology and Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA

Integrin and growth factor signaling cooperatively activate MAP kinase to regulate gene expression and cell behavior. Although mechanical cues such as cell adhesion are known to regulate integrin and growth factor signaling, how mechanical force could elicit effects on cell signaling has yet to be defined. We used a combination of pharmacological and genetic approaches together with a force-measurement technique called traction force microscopy to delineate the molecular mechanisms whereby mechanical force could influence ERK signaling. We showed that application of an exogenous force facilitates focal adhesion maturation and cell spreading through enhancement of β1 integrin clustering. Ectopic clustering of β1 integrin through expression of a β1 integrin self-clutering mutant also drove focal adhesion maturation and cell spreading in association with increased ERK activation and cell force generation, suggesting that a functional link between focal adhesions, ERK activity and force may exist. Consistently, we documented a correlation between Rho and ROCK activity, focal adhesion and cell force generation and implicated ERK as a key feedback regulator in this signaling circuit. Indeed, we could show that ERK activity correlates with cell force generation and that inhibiting ERK represses cellular forces in part by decreasing ROCK expression. Additional studies using synthetic flexible polyacrylamide substrata suggest that normal cells employ an ERK-integrin-Rho signaling-force circuit and tune their tensional homeostasis to the mechanical properties of the matrix microenvironment to regulate their growth and behavior. We also found that aberrant stiffening of the tissue microenvironment or uncoupling of the cellular tensional homeostasis signaling circuitry contributes to malignant behavior of an epithelium. (Supp HL6438801A1 & DODW 81XWH-05-1-330)

370

**Caveolin-1 Regulates Collagen Expression and Myofibroblast Differentiation in Normal Lung Fibroblasts Through MEK/ERK Signaling**

E. V. Tourkina,[1] M. Richard,[1] K. Charles,[2] R. M. Silver,[1] S. Hoffman[1]; [1]Rheumatology, Medical University of SC, Charleston, SC, [2]South Carolina State University, Orangburg, SC

Scleroderma (systemic sclerosis) is an autoimmune connective tissue disease that is frequently complicated by pulmonary fibrosis. Scleroderma lung fibroblasts, or myofibroblasts (SLF) overexpress collagen and contain high levels of activated MEK/ERK under baseline conditions. Our previous results demonstrate that caveolin-1 depletion activates MEK/ERK and increases collagen expression in normal lung fibroblasts (NLF). To determine whether increasing caveolin-1 activity will have the opposite effect, NLF and SLF received the caveolin-1 scaffolding peptide, a treatment known to mimic caveolin-1 overexpression. Similar striking effects were observed in both cell types. The treatment completely blocked collagen accumulation while decreasing MEK/ERK phosphorylation about 50%. Similar results were obtained when adenovirus was used to overexpress caveolin-1 in each cell type. To determine whether caveolin-1 also regulates myofibroblast differentiation, we evaluated whether perturbation of caveolin-1 would affect expression of the myofibroblast marker alpha-smooth muscle actin (ASMA). Depleting caveolin-1 increased ASMA expression in NLF, but had no effect on the already high level of ASMA in SLF. Conversely, overexpressing caveolin-1 decreased ASMA levels in SLF, and had little effect on the low levels of ASMA in NLF. Our observations that low caveolin-1 levels are correlated with high collagen levels and MEK/ERK activation in vitro were confirmed in vivo in mice with bleomycin-induced lung fibrosis and in vitro in lung tissue samples collected from human patients with scleroderma lung disease and from normal individuals. Therefore, these observations suggest that in lung fibroblasts caveolin-1 regulates both collagen expression and myofibroblast differentiation.

371

**Growth Factor Receptor or Erk MAP Kinase Inhibition Rescues Dysfunctional 3D-Mammary Epithelial Acinus Assembly**

E. McArdle, E. Gilligan, L. Thornton, J. Murtagh, F. Martin; Conway Institute, University College Dublin, Dublin, Ireland

Primary mouse mammary epithelial cells supported on a laminin-rich ECM, form 3D-acinar assemblies consisting of single layers of polarised, quiescent cells surrounding a cleared lumen. Inhibition of JNK results in failure of cell polarisation, lumen clearance and formation of dysfunctional spheres filled with surviving cells. Our objective was to characterise the component cells of these dysfunctional spheres and understand the regulatory mechanisms determining their behaviour. We show that these cells exhibit low expression of and non-polarised distribution of tight junction proteins, low relative expression of E-cadherin but high N-cadherin expression, coupled with expressing smooth muscle actin and vimentin. These are characteristics of epithelial cells initiated on transition to a mesenchymal phenotype. These cells also showed high levels of EGF-receptor autophosphorylation and ERK and Bim phosphorylation. We show that the 3D-assemblies forming in the presence of the JNK inhibitor, SP600125, properly polarise and undergo lumen clearance if they are cultured without growth factor (EGF) or in the presence of an EGF receptor autophosphorylation inhibitor. However, these spheres are significantly smaller than normal. ERK phosphorylation and Bim phosphorylation levels are reduced under these conditions. In fact the presence of the ERK signalling pathway inhibitor, PD98059, also protects against the dysfunctionalising effects of the JNK inhibitor. Indeed, after exposure to JNK inhibiton and the formation of dysfunctional spheres, addition of PD98059 is sufficient to 'rescue' and trigger cell polarisation, lumen clearance and generation of a normal acinar structure. But again these spheres are significantly smaller than normal. Thus integrin activation by the ECM and JNK act to support anti-proliferative and pro-apoptotic influences that include suppressing ERK activation and supporting cell-cell contacts. Inhibition of JNK is sufficient to promote cell proliferation, cell survival and the expression of EMT-like characteristics.

372

**Tumor Necrosis Factor-α Induces VCAM-1 Expression via Activation of MAPKs and NF-κB in Human Tracheal Smooth Muscle Cells**

C. Yang, C. Lee; Pharmacology, Chang Gung University, Tao-Yuan, Taiwan Republic of China

TNF-α has been shown to induce the expression of adhesion molecules in airway resident cells and contribute to inflammatory responses. Here, the roles of mitogen-activated protein kinases (MAPKs) and NF-κB in TNF-α-induced expression of vascular cell adhesion molecule (VCAM)-1 were investigated in human tracheal smooth muscle cells (HTSMCs). TNF-α-enhanced expression of VCAM-1 protein and mRNA as well as phosphorylation of p42/p44 MAPK, p38, and JNK were significantly attenuated by inhibitors of MEK1/2 (U0126), p38 (SB202190), and JNK (SP600125). Transfection with dominant negative mutants of MEK1/2, ERK1, ERK2, p38, and JNK attenuated TNF-α-induced VCAM-1 expression. Furthermore, TNF-α-induced VCAM-1 expression was significantly blocked by a selective NF-κB inhibitor helenalin. TNF-α-stimulated translocation of NF-κB into the nucleus and degradation of IκB-α was blocked by helenalin, but not by U0126, SB202190, or SP600125. VCAM-1 promoter activity was enhanced by TNF-α in HTSMCs transfected with VCAM-1-Luc, which was inhibited by helenalin, U0126, SB202190, and SP600125. Moreover, the resultant enhancement of VCAM-1 expression increased the adhesion of polymorphonuclear cells to monolayer of HTSMCs which was blocked by helenalin, U0126, SB202190, or SP600125. These results suggest that in HTSMCs, activation of p42/p44 MAPK, p38, JNK and NF-κB is essential for TNF-α-induced VCAM-1 expression.

373

**The p38 MAPK Pathway is Essential for Mallory Body Formation in vitro**

L. Nan, F. Bardag-Gorce, J. Li, B. A. French, S. W. French; Pathology, LA Biomedical Research Institute, Torrance, CA

To determine if the p38 MAPK pathway plays a role in Mallory body (MB) formation, primary cultures of hepatocytes from DDC-primed mice were studied. The DDC-primed hepatocytes were MB free when isolated and began to enlarge and elongate after a few hours of culture. MBs began to form spontaneously after 2-3 days of culture and numerous MBs were formed after 6 days of culture. The p38 MAPK inhibitor (p38i) was added to the medium 3 hours after planting the cultures. No MBs formed in the cells treated with 100μM p38i for 6 days and the cells stayed polyhedral in shape just as they appeared prior to culturing and in the 3h culture. Western blot showed that the formation of phosphorylated p38 MAPK was completely blocked in DDC-primed hepatocytes by p38i treatment. Immunostaining of 6 day DDC-primed hepatocyte cultures with antibodies to p62 and phospho-p38 MAPK showed that phosphorylated p38 MAPK was concentrated within MBs. The immunostaining was also used to determine phosphorylated p38 MAPK expression in the livers of 4 different groups of mice: control mice; mice fed DDC for 10 weeks; mice fed DDC 10 weeks, then withdrawn 5 weeks; mice fed DDC 10 weeks, withdrawn 4 weeks, then fed DDC + chlormethiazole (CMZ) for 1 week. P38 was located in the cytoplasm of the hepatocytes and at the fringe of the MBs. These data indicate that activated p38 MAPK may be involved in the mechanism of MB formation by phosphorylating cytokeratins, which overload the ubiquitin-proteasome pathway leading to keratin aggresome formation. The p38 MAPK pathway may also be involved in MB formation by activating the transcription factors such as ATF-2. ATF-2 regulates the critical proteins involved in MB formation such as Ubiquitin B and p62.

374

**Directed Cell Fate by Engineered Spatial Patterns of BMP-2**

J. A. Jadlowiec,[1] E. D. Miller,[2] G. Fisher,[1] L. Weiss,[3] A. Waggoner,[1] P. G. Campbell[4]; [1]Molecular Biosensor and Imaging Center, Carnegie Mellon University, Pittsburgh, PA, [2]Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, [3]Robotics Institute, Carnegie Mellon University, Pittsburgh, PA, [4]Institute for Complex Engineered Systems, Carnegie Mellon University, Pittsburgh, PA

During development, morphogens (hormones) and their inhibitors form discrete temporal and spatial patterns within the extracellular environment, including the incorporation within discrete extracellular matrices (ECMs). Such hormone patterns, both concentration gradients and discrete periodic shapes, are proposed to direct cell fate determination and lineage progression. Experimental approaches to establish defined persistent spatial hormone patterns, required for systematic study of cell differentiation, have remained a significant challenge. We have developed an ink jet-based bioprinting technology to engineer persistent and defined spatial patterns of hormones biologically immobilized (solid-phase) on biomimetic substrates. Our objective was to demonstrate the ability to direct differentiation of primary human adult mesenchymal stem cells (hMSC) towards an osteoblastic lineage using 2-dimensional spatial patterns of solid-phase bone morphogenetic protein-2 (BMP-2). Our strategy utilized our ink-jet printing technology to create biologically-inspired solid-phase patterns of BMP-2, an important hormone during tissue development including

bone. We assessed cell response by detection of Smad signal transduction pathway activation based on the rationale that Smads are the "first responders" to receptor activation by BMP-2. We demonstrated that solid-phase BMP-2 is biologically active: Smad signaling was activated and downstream target genes were induced. We quantified Smad1 sub-cellular localization and determined that Smad1 was predominantly nuclear for cells on pattern, in register with printed BMP-2. Whereas, control cells (cells off BMP-2 pattern), exhibited mainly cytoplasmic Smad1. We conclude that Smads can be used to identify a patterned cell response to solid-phase patterns of BMP-2, and may be useful to predict changes in cell fate and response to spatial patterns of morphogens. Our long term goal is to engineer cell differentiation in register to a hormone stimulus in a temporally and spatially-defined manner.

375

**Bone Morphogenetic Protein 4 Alters Epithelial Ovarian Cancer Cell Morphology, Motility and Adhesion**

B. L. Theriault, M. W. Nachtigal; Pharmacology, Dalhousie University, Halifax, NS, Canada

**Introduction:** We identified a functional bone morphogenetic protein (BMP) signaling pathway in human epithelial ovarian cancer (EOC) cells. EOC cells can adhere to extracellular matrix (ECM) proteins through integrin receptors and focal adhesion proteins (FAPs). These proteins likely contribute to EOC progression by facilitating cell adherence to peritoneal surfaces. BMP4 treatment results in altered cell morphology characterized by increased cell spreading. *We hypothesize that BMP signaling plays a role in EOC morphology, adhesion and motility by remodeling cell-ECM interactions.* **Methods**: EOC cells were treated with BMP4 for up to 7 days. F-actin staining and vinculin co-localization was used to view cytoskeletal changes. Quantitative PCR (QPCR) assessed mRNA levels of integrin receptors, FAPs and ECM proteins. An *in vitro* wounding assay evaluated EOC motility in response to BMP4 or BMP4 plus Noggin (BMP inhibitor), while cell adhesion to various ECM proteins was observed using a tritium incorporation assay. An adenovirus expressing the constitutively active Type I BMP receptor (Ad-Alk3QD) was also used to transduce EOC cells. **Results**: BMP4-induced cell spreading correlated with increased stress fiber formation and colocalization of vinculin to actin stress fibers; this morphological change was irreversible. QPCR showed increased integrin, FAP and ECM mRNA as early as 24hrs following BMP4 treatment. EOC cells demonstrated increased cell motility and adhesion in the presence of BMP4, while co-treatment of BMP4 and Noggin reduced motility to control levels. Ad-Alk3QD produced similar morphological changes as with exogenous administration of BMP4. **Conclusions**: BMP4 signaling induces changes in the molecules involved in cell morphology, adhesion and motility, translating into changes in cellular behavior. Future work will examine the intracellular signaling mediators responsible for producing this committed morphological change, enhancing our understanding of how BMP4 signaling may contribute to EOC biology.

376

**Dual Regulation of Type IV Collagen by Smad1 in Diabetic Nephropathy:TGF-b and BMP4**

H. Abe,[1] T. Tominaga,[1] T. Matsubara,[2] K. Nagai,[2] T. Takahashi,[1] H. Arai,[2] T. Kita,[2] T. Doi[1]; [1]University of Tokushima, Tokushima, Japan, [2]Kyoto University, Kyoto, Japan

We previously reported that advanced glycation end-products (AGEs) transcriptionally upregulate the type IV collagen (Col4), a key molecule for diabetic nephropathy. To identify a specific transcriptional factor, we used a yeast one-hybrid system to isolate a clone that encodes a specific binding protein from a mouse mesangial cell cDNA library. We identified this clone as the cDNA that encodes Smad1. We confirmed the binding of Smad1 to the Col4 promoter in vivo using chromatin immunoprecipitation (ChIP) assay. Transfection of Smad1 induced 23-fold increase of transcriptional activity of Col4 in COS7 cells. Smad1 is transcriptionally upregulated by AGEs in a time-dependent manner in mouse mesangial cells. Similarly, the levels of Col4 mRNA increased in parallel with the upregulation of Smad1 transcripts. Although Smad1 is an essential gene for renal development and is little expressed in normal adult glomeruli, Smad1 is significantly induced by AGEs stimulation. Moreover, knock-down of the Smad1 by antisense morpholino blocked the excessive collagen production. As Smad1 is generally known to transduce BMP4 signaling, we also demonstrated that AGEs induced BMP4, Smad1, and Col4 mRNA and protein expressions in mesangial cells. TGF-b neutralizing antibody or Noggin (a BMP antagonist) inhibited these inductions. Furthermore, Smad1 and BMP4 were highly expressed in mouse diabetic nephropathy. These results suggest that the modulation of both TGF-b/Smad1 and BMP4/Smad1 signaling is deeply responsible for initiation and progression of diabetic nephropathy and that blocking of Smad1 signaling pathway may be beneficial for diabetic complications.

377

**Tenascin-C EGF-Like Repeats - Novel Matrikine Ligands for EGFR**

A. V. Iyer,[1] K. T. Tran,[1] L. Richardson,[2] C. W. Borysenko,[1] H. C. Blair,[1] I. Bahar,[3] L. Griffith,[2] A. Wells[1]; [1]Department of Pathology, University of Pittsburgh, Pittsburgh, PA, [2]2School of Engineering Biological Engineering Division and Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, [3]Department of Computational Biology, University of Pittsburgh, Pittsburgh, PA

Select epidermal growth factor (EGF)-like repeats (EGFL) of human tenascin cytotactin can stimulate EGF receptor (EGFR) signaling. However, this activation requires micromolar concentrations of soluble EGFL in contrast to subnanomolar concentrations of classical growth factors such as EGF. To elucidate the underlying mechanism of this altered EGFL binding phenomenon, and assess if such binding resulted in distinct biochemical/biophysical responses as compared to prototypical growth factors, we recombinantly expressed 14$^{th}$ EGFL (Ten14) in monomeric form and tested them on murine fibroblasts over-expressing EGFR. Radio-ligand binding assays showed that Ten14 did not bind stably to EGFR, and there was no significant internalization of Ten14 over either the short or long term, both in contrast to EGF, suggesting altered binding dynamics for Ten14. Surface plasmon resonance analysis yielded a $K_D$ of 97μM for Ten14, approximately three logs higher than for EGF. We modeled Ten14 using structure prediction servers to produce a tight EGF-like molecule with truncated loops. Analysis of the generated bound structure suggested both Ten14 and EGFR have much more mobility in the binding pocket compared to EGF/TGFα, due to the absence of key high-affinity interactions which likely account for the low affinity binding of Ten14, and implies a flexibility enabling ligandation of receptor even when EGFL are embedded within the extracellular matrix. Such matrix-constrained presentation of EGFL would likely result in compartmentalization of EGFR on the cell surface, where PLCγ1 is predominantly activated, resulting in enhanced cell migration. Indeed, we observed sustained migration over 2D surfaces at levels of Ten14 that failed to stimulate proliferation, concomitant with potent PLCγ1 and M-calpain signaling, and weak activation of ERK/MAPK, p90Rsk and Elk1. Thus, this comprehensive study puts forth a novel mode of action for EGFL signaling through EGFR, and how this impacts cellular behavior.

378

**Endotoxin-Induced IL-6 and iNOS/NO Expression in Non-Immune Mouse Mammary Epithelial Cell Lines**

S. W. Maalouf,[1] R. S. Talhouk,[2] F. L. Schanbacher[1]; [1]Animal Sciences, Ohio State University, Wooster, OH, [2]Biology, American University of Beirut, Beirut, Lebanon

Recent findings link sustained inflammation to chronic disease or cancer in epithelia. Although inflammation in immune cells shows coordinate regulation of inflammatory respondents interleukin-6 (IL-6) and nitric oxide (NO) by nuclear factor kappa B (NFkB), little is known about such inflammatory responses in non-immune cells of epithelia. This study investigated IL-6 secretion, NO production, and induction of nitric oxide synthase (iNOS) relative to cell types and substratum during induced inflammation in mammary epithelia. Two mammary cell lines (CID-9 =heterogeneous (secretory epithelial, myoepithelial and fibroblast) cells; and SCp2 =subculture of CID-9 predominantly secretory epithelial and lacking myoepithelial cells) were grown with differentiating hormones on plastic +/-EHS extracellular matrix (ECM) before inducing inflammation with bacterial endotoxin (ET; 0-100ug/ml). ET-induced inflammatory responses were monitored by IL-6 secretion (ELISA immunoassay), NO production (Griess reaction assay), and IL-6 and iNOS mRNA expression (real-time PCR). Both cell types showed early ET dose-dependent induction of IL-6 secretion that plateaued by 6h with maximal IL-6 secretion 2-3-fold higher in CID-9 (ca 600 pg/ml) than in SCp2, especially on ECM. IL-6 mRNA peaked by 3h in CID-9 regardless of substratum. NO did not increase until 12h after ET treatment and continued throughout the 48h culture, with little or no difference between substratum or ET dose for either cell line. iNOS mRNA peaked at 3-6h and declined sharply thereafter in SCp2 but was sustained over 3-12h in CID-9 cells, with higher iNOS mRNA for SCP2 on ECM. The mutual upregulation with differences by cell type for IL-6 and iNOS in ET-treated mammary cells suggest coordinate but poorly understood differential regulation of inflammatory responses (and role for NFkB therein) by respondent and epithelial cell types or differentiation state, for which mammary epithelial cells are a good model.

## Cell-Cell Interactions I (379-393)

379

**Regulation of Connexin43 Expression and Function by Calreticulin**

M. Aghasi,[1] E. Kardami,[2] N. Mesaeli[1]; [1]Division of Stroke and Vascular Disease, Winnipeg, MB, Canada, [2]Institute of Cardiovascular Sciences, Winnipeg, MB, Canada

Gap junction channels between neighboring cells facilitate cell-cell communication and influence different processes in the cells, including proliferation and differentiation. Connexins are the building blocks of gap junctions. They are synthesized and folded in the endoplasmic reticulum and oligomerized in their way to the cell membrane. They have short half life and after internalization undergo degradation via both proteasome and lysosome. To date, no chaperons have been identified involving in the folding of connexin43. Calreticulin (CRT) as an endoplasmic reticulum resident chaperon indirectly regulates expression and function of a variety of proteins in cell. To elucidate the role of CRT in the regulation of connexin43 expression and function we utilized wild type and CRT knockout mouse embryonic fibroblast cells. Our data showed significantly higher connexin 43 protein in the CRT knockout cells. We also showed that this increase in the protein level was due to activation (2 fold) of connexin43 promoter. Interestingly, there was lower gap junction density at the cell-cell junction and a significant decrease in the gap junction function (dye transfer) in the absence of CRT. Inhibition of proteasome activity resulted in increased connexin43 localization to the plasma membrane and vesicular structures in the CRT knockout cells. Furthermore, inhibiting protein degradation via proteasome resulted in a significant decrease in connexin43 promoter activity while blocking lysosome activity had no effect. We conclude that in the absence of CRT Connexin43 is more sensitive to proteasome degradation and it would be internalized faster resulting in decreased gap junctional communication. Moreover, enhanced proteasome activity in CRT knockout cells stimulates connexin43 promoter activity.

380

**CLIC4 Plays Key Role in the Interaction Between Neural Photoreceptors and Retinal Pigment Epithelial Cells**

J. Chuang, C. Sung; Ophthalmogy, Dyson Vision Research Institute, Weill Med College of Cornell University, New York, NY

Retinal pigment epithelium (RPE) is part of the outermost layer of retina, sandwiched between the photoreceptor and choriocapillaries. Tight junctions formed between RPE cells prevent passive diffusion of molecules and allow the RPE to serve as the blood-retina barrier. The apical surfaces of RPE cells contact the subretinal space, which contains interphotoreceptor matrix and the distal tips of photoreceptor outer segments (OS). The basolateral surfaces interact with Bruch's membrane and choriocapillaries. In addition, RPE cells are professional phagocytic cells, participating in the diurnal phagocytic removal of spent photoreceptor OS tips. The development of RPE and photoreceptor is orchestrated by a two-way communication between the RPE and the neural retina; they mature around the same time postnatally. Unfortunately, the intimate relationship between RPE and photoreceptor interface is hard to model in vitro. By immunohistochemistry, we found that CLIC4, a member of the family of intracellular chloride channels (CLICs), was highly abundant in the apical microvillar processes of RPE. CLICs are a family of closely-related proteins sharing high homology across approximately 220 amino acids. Previous studies showed that CLIC4 generated an outwardly rectifying Cl- conductance upon reconstitution in a planar lipid bilayer, however, conductance was very low. We sought to study CLIC4's role in RPE in vivo by loss-of-function approach using an in vivo transfection method that delivers plasmids into the RPE layer of animals. RPE cells with CLIC4 suppressed by CLIC4-siRNA but not control-siRNA exhibited profound changes in cytoskeleton arrangement and protein expression pattern. More interestingly, the structural remodeling of RPE was associated with the retinal detachment and photoreceptor atrophy. We propose that CLIC4 is a key molecule involved in cytoskeletal organization of RPE, and critical for the interface between RPE-neural retinas.

381

**Cellular Regulation of the Hematopoietic Stem Cell Niche**

J. M. Gillette, J. Lippincott-Schwartz; Nichd, cbmb, National Institutes of Health, Bethesda, MD

The defining characteristics of stem cells make them remarkable. Not only do they have extensive proliferative potential, but they also have an ability to differentiate into multiple cell types. It is becoming increasingly clear that stem cell functions are regulated by both intrinsic stem cell factors and neighboring differentiated cells. This unique microenvironment is referred to as the stem cell niche. Hematopoietic stem cells (HSC),

which give rise to the blood and immune cell lineages, reside in the bone marrow. Recent studies have demonstrated osteoblasts, the bone forming cells, as critical components of the HSC niche. However, the cellular interactions between HSC and osteoblasts are not well defined. We utilized live cell imaging techniques to observe the physical relationship between HSC and osteoblastic cells cultured together. Studies carried out with human $cd34^+$ cells labeled with FM dyes in the presence of osteoblastic stromal cells expressing fluorescent proteins show a number of interesting interactions between the cells. Imaging began within 10 minutes of co-culture and a series of Z stack images were taken every 10 minutes for a 16 hour period. Time lapse monitoring displayed HSC attach to the osteoblastic monolayer within 20 minutes. Although attachment is maintained to one osteoblast, our imaging data suggest that HSC migrate on the osteoblastic surface for distances up to 10µm. Additionally, immunofluorescence experiments using the cd34 surface molecule of HSC detected clusters of progenitor cells lodged at specific osteoblastic sites suggestive of HSC proliferation. In combination these data demonstrate that the *in vitro* model system is valuable for observing the dynamic cellular interactions that occur between HSC and the osteoblastic 'niche' microenvironment.

382

**ß-actin is Locally Translated and Accumulated at the Cell Periphery and Cell Contacts**

A. J. Rodriguez, S. M. Shenoy, R. H. Singer, J. Condeelis; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

We developed a live cell imaging technique demonstrating that ß-actin mRNA, its sites of translation, and ß-actin protein were spatially correlated at the periphery of C2C12 mouse myoblast cells. However, in order to determine how ß-actin is locally translated and the newly synthesized protein sorted, a live cell imaging technique is required to correlate the distribution of translating ß-actin mRNA with the trafficking of newly synthesized ß-actin protein. ß-actin mRNA was actively translated in both the perinuclear cytoplasm and at the cell periphery. In contrast, ß-actin mRNA lacking the zipcode, a sequence necessary and sufficient for its localization to the cell periphery, was unable to translate there, confirming that the zipcode determines the sites of localized translation. At regions of cell-cell contact, ß-actin mRNAs were actively translating suggesting that these are microenvironments where translational repression was relieved. Pulse-chase staining showed that newly synthesized ß-actin protein accumulated at the leading edge of isolated cells or at cell-cell contacts. However, ß-actin synthesized from an mRNA lacking a zipcode sequence failed to accumulate either at the leading edge of isolated cells or at cell-cell contacts. Therefore, enrichment of newly synthesized protein at these sites was dependent on mRNA localization. These data demonstrate that the site of translation of a protein is an important component of its final disposition. This work was supported by NIH grants CA100324 to JC, AR41480 to RHS, and AR4148012S1 to AJR.

383

**Role of Epithelial CD44 in Regulation of PMN Transepithelial Migration**

K. Zen, C. A. Parkos; Pathology, Emory University, Atlanta, GA

Neutrophil (PMN) migration across mucosal epithelium during inflammatory episodes is a multistep process involving many cell surface molecules and signaling events. To identify epithelial ligands for migrating PMN, we screened a panel of antibodies generated against epithelial cells for inhibition of PMN transepithelial migration in the basolateral to apical direction. We identified a mAb termed gamma-35 that inhibited PMN transepithelial migration and strongly labeled the apical surface of intestinal epithelial cells. The expression of the antigen recognized by gamma-35 was significantly increased after exposure of epithelial cells to INF-γ. Microsequence analysis of the antigen purified from epithelial cells identified it as an epithelial-specific CD44 isoform. This protein is a highly glycosylated 150 kD membrane protein that, unlike other CD44 isoforms, is not expressed on PMN. MAb gamma-35 had no inhibitiory effects on PMN migration across acellular filters, suggesting the inhibition of migration is mediated by blockage of epithelial CD44. Kinetic studies of PMN transepithelial migration and immunofluorescencce localization experiments demonstrated that mAb gamma-35 blocked detachment of PMN from the apical surface of epithelial monolayers at a late stage of transmigration and suggest that epithelial CD44 is involved in regulating PMN detachment. Analyses of supernatants from transmigration assays revealed the presence of soluble epithelial CD44 following PMN transmigration, suggesting that shedding of epithelial CD44 might play a role in regulating PMN detachment from epithelia. In support of this hypothesis, both the shedding of epithelial CD44 and PMN transepithelial migration were blocked by mAb gamma-35 and MMP inhibitors. Taken together, these studies suggest that epithelial CD44 regulates PMN detachment during transepithelial migration and may be a novel target for anti-inflammatory therapies.

384

**Differential Association of Tyrosine 421- and 466-Phosphorylated Cortactin with Cell-Cell Contacts and Cytoskeleton in Corneal Endothelial Cells**

N. Savion, L. Kredy-Farhan; Goldschleger Eye Research Institute, Tel Aviv University, Tel Hashomer, Israel

Cortactin (p80/85), a phospho-tyrosine protein known as an actin-associated protein, was recently shown to be localized at cell-cell contacts in confluent epithelial and endothelial cells. Therefore, in the present study we explored the localization and association of the two tyrosine-phosphorylated variants of cortactin ($pY^{421}$ and $pY^{466}$) with the cytoskeleton and cell-cell contacts in cultured bovine corneal endothelial (BCE) cells. Cortactin expression and localization was determined by immunoblot and confocal microscopy, respectively, using specific antibodies against cortactin and its tyrosine-phosphorylated variants $pY^{421}$ and $pY^{466}$. Triton X-100 insoluble proteins were considered as cytoskeleton associated proteins. In confluent monolayer of BCE cells cortactin appeared as a fine continuous single line marking the cell-cell contacts close to but not in visible co-localization with the cortical actin-ring that appeared as a wide continuous ring in each cell. Only cortactin isoform p85 was observed phosphorylated on residues $Y^{466}$ or $Y^{421}$. This fine line of cortactin at cell-cell contacts was mainly phosphorylated at $Y^{466}$, while cortactin at the basal aspect of the cell, probably at focal adhesion sites, was phosphorylated at $Y^{421}$. Furthermore, upon cell-cell contacts formation, up-regulation of cortactin $pY^{466}$ was demonstrated. Cortactin was partially associated with cytoskeleton ptoteins (46±4% in the Triton X-100 insoluble fraction) and this fraction was phosphorylated on $Y^{421}$, while the soluble fraction was mainly phosphorylated on $Y^{466}$. In non-confluent cells, only cortactin $pY^{421}$ was found and localized at the leading edges of the cell, along the focal contacts (co-localized with FAK) and the actin stress-fibers. These results may suggest that in BCE cells cortactin $pY^{466}$ is involved in cell-cell contacts, while cortactin $Y^{421}$ is associated with focal adhesion sites. Furthermore, the non-tyrosine-phosphorylated cortactin molecules are preferentially associated with the cytoskeleton.

385

**Synchronization of the Beating of the Cardiomyocytes by the Heterologous Cells with the Agarose Microchamber System**

T. Kaneko, K. Kojima, K. Yasuda; Graduate School of Arts and Sciences, University of Tokyo, Tokyo, Japan

To understand the community effect in cardiomyocytes, the stepwise network formation of cells as the reconstructive approach using the on-chip agarose microchamber system with photo-thermal etching method was applied. In this system, the shapes of agarose microstructures were changed step by step with photo-thermal etching of agarose layer of the chip using a focused laser beam (wavelength: 1064 nm or 1480 nm) to increase the interaction of cardiomyocytes during cultivation. We tried to control the beating of two cardiomyocytes and the cardiomyocytes through the constitutive arrangement of the connection to the fibroblasts. It was observed that the beating of cardiomyocytes synchronized suddenly after dozens of minutes, after cardiomyocytes contacted physically. Furthermore, when the number of cardiomyocytes was increased step by step, the fluctuation of the beating intervals of the cardiomyocytes was stabilized. However, when the beating of cardiomyocytes was synchronized through the fibroblasts arranged between the cardiomyocytes, it was observed that the beating of cardiomyocytes synchronized after the synchronization and asynchronization of the beating repeatedly for dozens of minutes. These results indicate the importance of the community size and the heterogeneity of cells to stabilize their performance for making cell-network model for using cells for monitoring their functions like the tissue model.

386

**Ouabain Changes the State of Phosphorylation and Localization of Adhesion and Signaling Proteins**

D. Flores-Benitez, C. E. Flores-Maldonado, I. Larre, F. García-Santín, L. Shoshani, M. Cereijido, R. G. Contreras; Departamento de Fisiología, Biofísica y Neurociencias, CINVESTAV-IPN, D.F., Mexico

In a previous work we described a P→A mechanism that transduces occupancy of the $Na^+,K^+$-ATPase (P = pump) by ouabain, into changes in phosphorylation and retrieval of cell-cell and cell-substrate attaching (A) molecules, thus causing a release of the cell from the monolayer. P→A involves activation of protein tyrosine kinases (PTKs) and of Extracellularly Regulated Kinases (ERK1/2), as well as an increase in the cell content of $p190^{Rho\text{-}GAP}$, a Rho GTPase promoter. Here we observe that one of the PTKs activated by ouabain is c-Src, whose inhibition with PP2 prevents cell detachment. The activation of PTKs is necessary for the phosphorylation of ERKs, a step that can be blocked by genistein. Phosphorylated ERKs are in turn necessary for the inactivation and decrease of cell content of RhoA; this last effect can be prevented by the ERK inhibitor UO126. Pursuing the effect of ouabain on cell detachment, we observe that inhibition of c-Src (with PP2) or ERK1/2 (with PD98059) prevent retrieval of occludin, β-catenin and focal adhesion kinase (FAK), which are conspicuous members of tight, adherens and focal junctions respectively. Retrieval of β-catenin appears to be associated to an increase of tyrosine phosphorylation mediated by ERK1/2. FAK instead is dephosphorylated on tyrosine 397, a process which is also mediated by c-Src.

387

**T Cell Exosomes Enhance Lipid Accumulation in Human Macrophages**

S. Dadsetan, L. Zakharova, A. F. Fomina; Physiology and Membrane Biology, University of California, Davis, Davis, CA

Activated human T cells release vesicles, termed exosomes, originated from endocytic compartments. We reported previously (Fomina et al. 2003. *Exp. Cell Res.* 291:150-66) that exosomes substantially differ in lipid composition from the plasma membrane as they are composed of cholesterol enriched raft membranes and expose phosphatidylserine at their outer membrane leaflet. The development of macrophage-derived foam cells that contain massive amounts of cholesterol esters is a hallmark of atherosclerotic lesions and is mediated by scavenger receptors that recognize phosphatidylserine. Because activated T cells are abundant in atherosclerotic lesions we explored whether T cell exosomes would affect lipid accumulation in macrophages. Resting T lymphocytes were purified from the peripheral blood of healthy volunteers and were activated in vitro with phytohemagglutinin P to stimulate exosome release. Exosomes were isolated from T cell culture supernatants using high speed centrifugation. Pellets obtained from T cell cultures displayed positive staining with cholera toxin B and annexin V and were composed of vesicles with the size and shape typical for T cell exosomes. The preparation of T cell exosomes was added to the culture of differentiated human macrophages. After overnight incubations, macrophages were fixed and stained with Oil red O, a lipoprotein-sensitive dye. Subsequent confocal imaging study revealed that macrophages incubated with T cell exosomes were enlarged in size and accumulated a significantly larger amount of lipoproteins than control cells. These results support the hypothesis that exosomes released by activated T cells are internalized by human macrophages resulting in enhanced macrophage growth and lipid accumulation. Research described in this abstract was supported by Philip Morris USA Inc. and Philip Morris International.

388

**Formation of Rosettes between Autologous Cultured Monocyte-Macrophages and Lymphocytes: Analysis by a Regression Model**

I. T. C. Novak, H. R. Cabral; Institute of Cell Biology, National University of Cordoba-Argentina. Faculty of Medicine, Cordoba-Argentina, Argentina

We found a selective cell-cell interaction with formation of rosettes between human blood monocyte-derived macrophages and lymphocytes (MLRs) in samples harvested under certain conditions from total leukocyte cell cultures (Rev Fac Cienc Med Cordoba 50 (2):25-6, 1992; Mol Biol Cell 7:S, 603a, ASCB 1996; Mol Biol Cell 8:S, 414a, ASCB 1997; Am J Hematol 60: 285-8, 1999). We found that the lymphocytes that joined to monocyte-macrophages in MLRs are T CD4+, and this phenomenon is time and cell density dependent. We found that gangliosides inhibited it partially (Mol Biol Cell 12:S, 477a, ASCB 2001). We postulated that antigen presentation would be involved in MLRs and we found that CD66 monoclonal antibodies recognize neutrophil material phagocitized by macrophages that formed immunological synapses and MLRs (Mol Biol Cell 13:S, 393a, ASCB 2002). Co-cultured naive purified macrophages and lymphocytes produces few MLRs and immunologic synapses but if antigens are added both phenomena are stimulated. (Mol Biol Cell 14:S, 458a, ASCB 2003). Inhibitors of antigen processing and presentation such as Brefeldin A (BFA) and Chloroquine inhibited these phenomena (Mol Biol Cell 15:S, 94a, ASCB 2004). We postulated that the cell-cell interaction in MLRs may be culture-time dependent. Here, we study the relation of MLRs and culture-time employing a Regression Model to obtain the equation of adjusted model. Methods: autologous human total leukocyte cultures from 30 healthy donors. Samples were harvested at various times: 15, 24, 48, 72, 96 and 120 hours of culture and centrifugated and performed as previously reported. Results: (R square 0.964), ANOVA for

Regression ($p<0.0001$). The equation obtained was: RML= -0.402 + 0.109 culture hours. These results support our hypothesis that a linear relation between MLRs formation and culture-time occurs.

389

**A Liquid Miscibility Model of Tumor-Stromal Interaction in Prostate Cancer**

D. Jia, J. Parron, M. Nurzia, J. Rosenthal, S. Kesler, R. A. Foty; Surgery, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ

We present a model in which prostate cancer-stromal interaction can be modeled as a liquid miscibility/immiscibility phenomenon, rooted in the same thermodynamic principles that govern the interactions between simple liquids. We apply an analogy of the high energy interaction between ethanol and water resulting in miscibility of the two phases, or the lower energy of interaction between oil and water resulting in immiscibility, to demonstrate that the model can differentiate between such interactions between mixtures of prostate cancer (CaP) and stromal cells. We show that when differentially-labeled non invasive AT-2 or invasive MLL cells are mixed with rat prostatic fibroblasts (RPF), AT-2 cells segregate from RPF cells, while MLL cells remain intermixed, suggesting that MLL-RPF interaction is of higher energy than that of AT-2 and RPF. A switch in the composition of the prostatic stroma from smooth muscle (SMCs) to fibroblastic is a hallmark of malignant transformation in CaP. We mixed MLL cells either with rat prostatic SMCs or RPF cells and showed that MLL cells segregate from SMCs but intermix with fibroblasts. This indicates that MLL cells have higher affinity for prostatic fibroblasts than for SMCs and is consistent with the observation that the stroma of more aggressive prostate cancers is predominantly fibroblastic. Further, we show that altering the adhesive relationship between tumor and stromal cells can shift a high affinity invasive interaction to one of lower affinity resulting in a shift from cell intermixing to segregation. We treated MLL cells with MEK inhibitors (MEKi) and showed that treatment restored the ability of MLL cells to assemble fibronectin into a fibrillar matrix leading to increased cell-cell cohesion and segregation from RPF cells. Our model provides a basis for exploring epithelial-stromal interaction in an easily manipulated *in vitro* system.

390

**A Neuronal Membrane Protein Required for Dendrite and Glial Process Extension in *C elegans***

M. G. Heiman, S. Shaham; Rockefeller University, New York, NY

Nervous system assembly and function rely on the coordinated development of complex cell shapes. The *C. elegans* amphid, the primary sensory organ of the worm, is a model for neuron and glial cell morphogenesis. It consists of 12 sensory neurons that each extend an axon and a dendrite, and two glia, one of which extends a process that is tightly bundled to the dendrites. Although axon development is well-studied, little is known about how dendrites or glial processes form. To understand dendrite and glia morphogenesis, we conducted a visual screen for mutants with defective amphid morphology. We identified mutants in two genes, *dex-1* and *dyf-7*, in which dendrites fail to grow, a novel phenotype we call Dex (*d*endrite *e*xtension defective). Notably, axons appear normal, indicating that dendrite growth requires different machinery than axon growth. In addition to shortened dendrites, Dex mutants display shortened glial processes. In some mutants, dendrites and glial processes are of variable lengths within a population, yet in a single amphid are always of equal length, revealing that dendrite and glial process extensions are coupled. We cloned *dyf-7* and found that it encodes a putative membrane protein with a conserved extracellular domain. *dyf-7* is expressed in neurons and required at the time of dendrite extension. Promoter-swap experiments show it can act in neurons or, suprisingly, in glia. A truncated DYF-7 protein, predicted to be secreted, is capable of rescuing *dyf-7* mutants. Thus, DYF-7 likely acts in the extracellular milieu. To understand how *dyf-7* promotes dendrite growth, we have established an optical cell-marking system to visualize dendrite extension in real-time. Because DYF-7 contains a conserved domain implicated in neuronal morphogenesis in other systems, amphid development may reflect a conserved means of sensory organ formation.

391

**TGFβ2 Inhibits Angiogenesis in a Novel *In Vitro* 3-D Model of the Respiratory Mucosa**

H. G. R. Thompson, D. T. Truong, C. K. Griffith, S. C. George; Biomedical Engineering, University of California, Irvine, Irvine, CA

Asthma is a chronic disease of the bronchial airway resulting in airway constriction. Local factors repair injured tissue, resulting in airway remodeling including vascularization. The current study developed an *in vitro* 3-D model of the human airway to analyze capillary sprout formation and network complexity in response to physiologic concentrations of TGFβ2 seen under conditions of epithelial injury. Many factors are known to influence angiogenesis and wound healing, including members of the VEGF and TGFβ families. Previous findings in our lab indicate that primary human bronchial epithelial cells (NHBE) increase soluble active TGFβ2 in response to mechanical denudation. The exact nature of TGFβ2 involvement is still under debate with conflicting results. The current model modifies a previous tissue construct in which primary human umbilical cord vein endothelial cells (HUVECs) were seeded onto cytodex microcarrier beads suspended in fibrin. Primary human lung fibroblasts (NHLF) were plated atop the fibrin gel at a distance supporting diffusion of soluble mediators. Our modification adds a differentiated primary NHBE cell layer seeded on a permeable Transwell membrane cultured at an air-liquid interface. Characterization of angiogenesis is defined by total capillary network length, number of vessel sprouts per bead, and number of vessel segments. TGFβ2 and VEGF concentrations were assessed by ELISA This *in vitro* tissue construct models many cell-cell interactions of lung tissue *in vivo*. In particular, NHLFs support capillary development, and NHBE induce further capillary network formation, supporting the role of VEGF in vascularization. Exogenous addition of VEGF further induces tube formation and network length. While TGFβ2 inhibits tubulogenesis in a dose-dependent manner, it also increases secreted VEGF suggesting that TGFβ2-inhibited angiogenesis is not regulated via the MEK/ERK pathway.

392

**Differential Adhesion Specifies Topology of Pseudoislets of pancreatic beta and alpha cells**

D. Jia, D. DaJusta, V. Peng, K. Bennett, R. A. Foty; Surgery, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ

Islet cell transplantation represents a promising cure for Type I diabetes. However, human islets available for transplantation are in extremely limited supply. Identifying an alternative source of cells and their constitution into functional islets is an important goal for the treatment of this disease. Cellular organization in pancreatic islets is evolutionarily conserved, non-random, and perturbed in diabetes suggesting that islet topology influences function. Understanding the mechanisms underlying the establishment of islet topology and its impact on function will facilitate the

design process. Isolated islet cells self-assemble in vitro into pseudoislets with the same cell type organization as native islets. It is widely held that differential adhesion contributes to the concentric segregation of islet cell types in vitro and in vivo. This postulate has never been directly tested since, until recently, no method existed which could rigorously quantify differences in adhesive properties of islet cells. We developed tissue surface tensiometry specifically for this purpose. We show that (1) randomly mixed immortalized beta (INS-1, MIN6) and non-beta (alpha-TC) cells sort-out in vitro in configurations resembling those of native islets with INS-1 and MIN6 cells forming the core and enveloped by alpha-TC cells; (2) aggregates of internally-segregating INS-1 and MIN6 cells are substantially more cohesive (2.52 dynes/cm, 1.35 dynes/cm, respectively) than those of the externally-segregating alpha-TC line (0.63 dynes/cm); (3) altering the cohesivity of alpha-TC cells results in phase reversal further confirming that differential adhesion specifies in vitro pseudoislet topology; (4) topology is not specified by differential expression of either cadherins or N-CAM but rather may be associated with differential capacity for fibronectin matrix assembly. Collectively the data strongly indicate that differential adhesion is the physical mechanism underlying the non-random organization of cells in pancreatic islets.

393

### Endothelial Cells Increase Tumor Cell Invasion into 3D Collagen Gels

C. T. Mierke, J. Pauli, P. Kollmannsberger, B. Reischl, W. H. Goldmann, B. Fabry; Center for Medical Physics and Technology, Biophysics, University of Erlangen-Nuremberg, Erlangen, Germany

Endothelial cells cultured as a monolayer on a gel or membrane are known to form a barrier against leukocyte and fibroblast cell invasion into the underlying extracellular matrix. Here we investigated whether endothelial cells can also form a barrier against tumor cells. We developed a 3D collagen gel assay to analyze invasion depth and number of invaded tumor cells in the presence or absence of a closed endothelial monolayer. We tested 13 tumor cell lines isolated from different organs (breast, skin, lung, brain, bladder and pancreas). Endothelial cells were isolated from human umbilical vein and used from 1$^{st}$ to 2$^{nd}$ passage. Tumor cells were stained with carboxyfluorescein diacetate and DNA-intercalating dye Hoechst 33342 to distinguish them from endothelial cells. Transmigration and invasion of tumor cells (seeded at 200 cells/sqmm) were analyzed after 3 days by computer-assisted optical 3D sectioning using a motorized fluorescent microscope. Images stacks were taken in 2 µm increments, and the invasion depths of each tumor cell was recorded. We quantified invasiveness for each tumor cell line in the presence and absence of an endothelial monolayer as average invasion depth (in mm) times number of invasive tumor cells per sqmm field of view. Except for T47D breast carcinoma cells, all tumor cell lines showed dramatically increased invasion in the presence of endothelial cells. Averaged over all cell lines, invasiveness was 1.88/mm ± 0.28 in the absence and 7.55/mm ± 1.25 in the presence of endothelial cells ($p < 0.001$). These results demonstrate that endothelial cells are unable to form a barrier against tumor cells and, surprisingly, even stimulate invasion of tumor cells, regardless of their tissue origin. These findings suggest a new role that endothelial cells may play in the process of tumor cell metastasis.

## Membrane Channels & Transporters I (394-418)

394

### Novel Functional Interaction between $Na^+/H^+$ Exchanger 1 and Tyrosine Phosphatase SHP-2

J. Xue,[1] D. Zhou,[1] H. Yao,[1] M. J. McConnell,[2] B. D. Gelb,[3] G. G. Haddad[4]; [1]Pediatrics, Albert Einstein College of Medicine, Bronx, NY, [2]Medicine, Mount Sinai School of Medicine, New York, NY, [3]Pediatrics and Human Genetics, Mount Sinai School of Medicine, New York, NY, [4]Pediatrics and Neuroscience, Albert Einstein College of Medicine, Bronx, NY

Besides being a $pH_i$ regulator, $Na^+/H^+$ exchanger 1 (NHE1) has recently been postulated as a membrane scaffold to assemble protein complexes and coordinate various signaling pathways. The aim of the current study is to uncover NHE1 interactive proteins and study their functional implications. NHE1 interactive partners were screened in the mouse brain using a signal transduction antibodyarray. Ten out of 400 tested proteins appeared to be potentially associated with NHE1. These partners have been shown to be involved in either cell proliferative or apoptotic pathways. The interactions between NHE1 and tyrosine phosphatase SHP-2, Bin 1 or heat shock protein 70 were reciprocally confirmed by co-immunoprecipitation. Furthermore, the functional significance of the association between NHE1 and SHP-2 was investigated for intracellular pH ($pH_i$) regulation and for cell proliferation using the fluorescent dye BCECF and [$^3$H]-thymidine incorporation respectively. Our data revealed that cells with SHP-2 overexpression exhibited a higher steady-state $pH_i$ and a faster, $Na^+$-dependent $pH_i$ recovery rate from acid load in the HEPES buffer. Moreover, the inhibition of cell proliferation by NHE1 blockade (HOE642) was diminished in these SHP-2 overexpressing cells. Taken together, our findings demonstrate that SHP-2 is not only associated with NHE1 but also positively modulate NHE1 functions such as $pH_i$ regulation and cell proliferation. This work was supported by National Institutes of Health grants PO1HD32573 and RO1NS037756 to Gabriel G. Haddad.

395

### Annexin II Light Chain p11 is an N-Terminal Auxiliary Subunit of Acid-Sensing Ion Channel ASIC1a

E. Donier,[1] F. Rugiero,[1] K. Okuse,[2] J. N. Wood[1]; [1]Molecular Nociception Group, Department of Biology, University College London, London, United Kingdom, [2]Department of Biological Sciences, Imperial College, London, United Kingdom

Acid-Sensing Ion Channels (ASICs) are voltage-independent $H^+$-gated ion channels belonging to the amiloride-sensitive DEG/ENaC superfamily of receptor channels. They have been implicated in a wide variety of physiological functions, such as nociception, mechano-transduction, learning, memory and fear behaviour, visual transduction and cochlear function. Recently homomeric ASIC1a channels were shown to be a major non-voltage-gated pathway for $Ca^{2+}$ entry in cells and $Ca^{2+}$ overload through ASIC1a channels was proven to make a major contribution to hippocampal neuronal damage in stroke. Therefore, the search for factors regulating ASIC1a function has become more pressing with respect to the development of selective pharmacological agents. We have used a rat dorsal root ganglion cDNA library in a yeast 2-hybrid assay to identify proteins that interact with ASICs. We found that the annexin II light chain p11 physically interacts with the N-terminus of ASIC1a but not other ASIC isoforms. Co-expression of p11 and ASIC1a in CHO-K1 cells, lead to a 2-fold increase in expression of the ion channel at the cell membrane, as judged by membrane-associated immunoreactivity and cell surface biotinylation. Consistent with these findings ASIC1a peak currents in transfected CHO-K1 cells were upregulated 2-fold in the presence of p11, whilst ASIC-3 mediated currents were unaffected by p11 expression. Neither the pH dependence of activation or rates of desensitisation were altered by p11, suggesting its primary role in regulating

ASIC1a activity is to enhance cell surface expression of ASIC1a. These data demonstrate that p11, already known to traffic members of the voltage-gated sodium and potassium channel families, as well as Transient Receptor Potential and chloride channels, also plays a selective role in enhancing ASIC1a expression.

396

**Transient Receptor Potential Thermo-Sensitive Channels Mediate Cytosolic Calcium Influx in Synoviocytes**

M. Y. Kochukov, T. A. McNearney, Y. Fu, Y. H. Ma, K. N. Westlund; University of Texas Medical Branch, Galveston, TX

The transient receptor potential (TRP) channels are important membrane sensors, responding to thermal, chemical, osmotic or mechanical stimuli by activation of calcium and sodium fluxes. In this study, we detected expression of three distinct TRP-channels and established their role in mediating cytosolic calcium ($[Ca^{2+}]_c$) response in tumor-derived SW982 synoviocytes and primary cultures of human synovial cells from patients with inflammatory arthropathies. Using Fura-2 ratio measurements while incubating cells in a temperature regulated chamber, we observed significant $[Ca^{2+}]_c$ elevation elicited by rapid changes in bath temperature, application of TRPV1 receptor agonists, capsaicin and resiniferatoxin, or a cold receptor stimulator, icilin. No changes in $[Ca^{2+}]_c$ were produced by menthol. Temperature thresholds for calcium response were determined to be 12 ± 1 ºC for cold and 28 ± 2 ºC for heat activation. Temperature increases or decreases beyond these thresholds resulted in a significant rise in the magnitude of $[Ca^{2+}]_c$ spikes. Observed changes in $[Ca^{2+}]_c$ were completely abolished in Ca-free media and resulted from direct Ca entry through TRP channels rather then by activation of voltage-dependent calcium channels. RT-PCR detected two heat sensitive channels, TRPV1 and TRPV4, and a cold-sensitive channel, TRPA1. No mRNA for TRPV3 or TRPM8 was amplified. The RT-PCR results support the data obtained with the $[Ca^{2+}]_c$ measurements. TRPV1 channels were also detected immunohistochemically with increases in staining after capsaicin. We propose that the TRP channels are functionally expressed in human synoviocytes and may play a critical role in adaptive or pathological changes in articular surfaces during arthritic inflammation. Supported by The Dana Foundation and NIH P01 NS011255-31.

397

**Protein Expression of G-Protein Inwardly Rectifying Potassium Channels (GIRKs) in Breast Cancer Cell Lines**

M. S. Dhar,[1] H. K. Plummer[2]; [1]Large Animal Clinical Sciences, University of Tennessee, Knoxville, TN, [2]Pathobiology, University of Tennessee, Knoxville, TN

Previous data from our laboratory has indicated that there is a functional link between the beta-adrenergic receptor signaling pathway and the G-protein inwardly rectifying potassium channel (GIRK1) in breast cancer cell lines and these pathways were involved in growth regulation of these cells (BMC Cancer, 2004 4:93). These previous studies indicated gene expression of GIRK channels. To continue these studies, we determined GIRK protein expression. Protein levels were determined by western blotting after isolation of membrane associated proteins. Expression of GIRK1 at the indicated molecular weight (MW) for the antibody (62 kDa) was seen in the breast cancer cell lines MDA-MB453 and ZR-75.1. In addition, GIRK1 expression was seen at a lower MW in the MDA-MB361, MDA-MB468, MCF-7, ZR-75.1 and MDA-MB453 cell lines. GIRK1 protein was not expressed in MDA-MB435 cells, corresponding to the lack of mRNA expression in previous research. GIRK1 protein expression was seen in MDA-MB468 cells, however mRNA expression was lacking in our previous studies. To prove that the lower MW protein was GIRK1, MDA-MB453 and MDA-MB468 cells were immunoprecipitated, and then separated by western blotting. The specific band on these gels was at the lower MW. We believe this to be post-translational modification of the protein. This is the first report indicating GIRK protein expression in breast cancer cells. To determine functionality, MDA-MB453 cells were stimulated with ethanol, known to open GIRK channels. Increases in GIRK1 protein expression were seen after 30 minute treatment with 0.12% ethanol. In addition, serum-free media completely inhibited GIRK1 protein expression. Our data indicates that there are functional GIRK channels in breast cancer cells and these channels are involved in cellular signaling. Research supported by a grant from Philip Morris USA Inc. / Philip Morris International.

398

**Mechanisms of ATP Release in Human Airway Epithelial Cells**

N. Cheng,[1,2] E. M. Schwiebert[1,2]; [1]Department of Physiology and Biophysics and of Cell Biology, Univ. of Alabama at Birmingham, Birmingham, AL, [2]Gregory Fleming James Cystic Fibrosis Research Center, Univ. of Alabama at Birmingham, Birmingham, AL

Secreted ATP is an important autocrine and paracrine signaling molecule in most if not all cells and tissues. Nevertheless, the cellular and molecular mechanisms of ATP release are understood poorly. Putative mechanisms fall into two broad categories: ATP-filled vesicles and ATP transporters/ channels. In particular, ATP-permeable anion channels such as “maxi” or large conductance anion channels, voltage-dependant anion channels (VDAC), connexin or gap junction hemichannels, and the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel. RNA interference is being used by our laboratory to assess the relative roles of the above anion channel candidates in flow-, mechanical-, calcium-, cyclic nucleotide-, and hypotonicity-induced ATP release. Low temperature- and tetanus toxin active fragment-induced block of vesicle trafficking and fusion is also used to assess the above. Taken together, we have identified roles for vesicle- and channel-mediated ATP secretion in basal and stimulated autocrine and paracrine purinergic signaling in airway epithelia. Future work seeks to identify the cellular mechanisms that remain intact in cystic fibrosis (CF) airway epithelia and participate in endogenous zinc and ATP co-secretion to stimulate apical P2X receptor channels independent or in addition to exogenously applied ligands.

399

**A Dynamic Role of Calbindin-D28K in TRPV5-Mediated Ca2+ Reabsorption**

T. T. Lambers,[1] F. Mahieu,[2] E. Oancea,[3] F. de Lange,[4] T. Voets,[2] D. Clapham,[3] B. Nilius,[2] J. G. Hoenderop,[1] R. J. Bindels[1]; [1]Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, [2]Physiology, KU Leuven, Leuven, Belgium, [3]Cardiovascular Dept, Children's Hospital and Harvard Medical School, Boston, MA, [4]Celbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

**Objectives;** TRPV5 and TRPV6 facilitate $Ca^{2+}$ influx in a variety of epithelial cells and are consistently co-expressed with the $Ca^{2+}$-binding proteins calbindin-$D_{9K}$ and/or -$D_{28K}$. Calbindins are thought to be involved in facilitated diffusion of $Ca^{2+}$ from the point of entry to the extrusion site, while simultaneously preventing cytotoxic high $Ca^{2+}$ levels. The aim of the present study was to investigate the role of calbindins in transepithelial $Ca^{2+}$ transport and their influence on the activity of TRPV5 and TRPV6. **Methods;** We investigated binding of calbindin-$D_{9K}$ and -

$D_{28K}$ with TRPV5/6 using pull-down and co-immunoprecipitation analysis. The translocation of calbindin-$D_{28K}$ towards the plasma membrane was assessed by cell fractionation techniques and Total Internal Reflection Fluorescent Microscopy (TIRFM). The functional effect of calbindins on TRPV5/6 activity was studied by $^{45}Ca^{2+}$ uptake and electrophysiological measurements. **Results;** TRPV5 associated with calbindin-$D_{28K}$ only when the intracellular $Ca^{2+}$ concentration was low due to BAPTA buffering. Furthermore, in the absence of $Ca^{2+}$ an increased calbindin-$D_{28K}$ abundance was detected in isolated plasma membranes. TIRFM revealed that calbindin-$D_{28K}$ translocates towards the plasma membrane when cells are treated with BAPTA-AM. $^{45}Ca^{2+}$ uptake in TRPV5-expressing MDCK cells was increased in the presence of calbindin-$D_{28K,}$ but not in the presence of a $Ca^{2+}$-insensitive calbindin-$D_{28K}$ mutant emphasizing the importance of the $Ca^{2+}$-binding EF-hand structures. The direct effect of calbindin-$D_{28K}$ on the activity of TRPV5 at a controlled $[Ca^{2+}]_i$ was investigated by uncaging $Ca^{2+}$ from the photolysable $Ca^{2+}$ chelator DMNP-EDTA. The $Ca^{2+}$-dependent inhibition of the TRPV5 current was similar in the presence of wild-type or $Ca^{2+}$-insensitive calbindin-$D_{28K}$. **Conclusions**; We propose a novel model in which translocation of calbindin-$D_{28K}$ towards the plasma membrane occurs at low intracellular $Ca^{2+}$ concentrations allowing association with TRPV5 to regulate the TRPV5 activity by buffering the local influx of $Ca^{2+}$.

400

### Recruitment of the Epithelial Ca2+ Channel TRPV5 to the Plasma Membrane is Dependent on Extracellular pH

T. T. Lambers,[1] E. Oancea,[2] D. Clapham,[2] J. G. Hoenderop,[1] R. J. Bindels[1]; [1]Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, [2]Cardiovascular Dept, Children's Hospital and Harvard Medical School, Boston, MA

**Objectives:** TRPV5, a member of the Transient Receptor Potential (TRP) superfamily is the gatekeeper facilitating $Ca^{2+}$ influx in epithelial cells of the kidney. A major physiological effect of extracellular pH is the regulation of membrane transport and regulation of renal $Ca^{2+}$ homeostasis. In the present study, we assessed whether the trafficking of TRPV5 is regulated by extracellular pH. **Methods:** Using Total Internal Reflection Fluorescent Microscopy (TIRFM) and biotinylation experiments the effect of extracellular pH on the recruitment of TRPV5 to the plasma membrane was investigated. After chemical bleaching with MTSET to irreversible inactivate membrane targeted TRPV5, the recovery was studied at different values of extracellular pH. The influence of extracellular pH on TRPV5-mediated $Ca^{2+}$ influx was investigated by $^{45}Ca^{2+}$ uptake experiments and modulators of vesicular trafficking were added during the uptake to provide insight into the mechanism of TRPV5 recruitment to the plasma membrane. **Results:** TRPV5-mediated $^{45}Ca^{2+}$ uptake was stimulated at an increased extracellular pH (pH 8.5), whereas at a decreased pH (pH 6.0) the TRPV5-mediated $^{45}Ca^{2+}$ uptake was diminished. An increase in extracellular pH resulted in a rapid and dramatically increased membrane-associated TRPV5-eGFP signal. Biotinylation experiments revealed that this rapid recruitment of intracellular TRPV5-containing vesicles led to insertion of active TRPV5 channels in the plasma membrane. Furthermore, the recovery after chemical bleaching with MTSET was elevated at alkali extracellular conditions. In addition, disruption of microtubules with nocodazole led to a decrease in the pH-stimulated TRPV5 mediated $^{45}Ca^{2+}$ uptake. **Conclusions:** pH-dependent recruitment of TRPV5 towards the plasma membrane is a novel way to regulate $Ca^{2+}$ transport in kidney and is dependent on microtubule mediated vesicular transport.

401

### Interleukin-1β Activates $Na^+$-$K^+$-$2Cl^-$ Cotransporter in Human Middle Ear Epithelia

J. Kim, J. Choi, J. Yoon, W. Namkung, E. Son, H. Park, M. Lee; College of Medicine Yonsei University, Seoul, Republic of Korea

Disruption of periciliary fluid homeostasis is the main pathogenesis of otitis media with effusion (OME), which is one of the most common childhood diseases. Although the underlying molecular mechanisms are unclear, it has been suggested that the altered functions of ion channels and transporters are involved in the fluid collection of middle ear cavity of OME patients. In the present study, we analyzed effects of the inflammatory cytokine IL-1ß on $Na^+$-$K^+$-$2Cl^-$- cotransporter 1 (NKCC1) in human middle ear cells. Intracellular pH ($pH_i$) were measured in primary cultures of normal human middle ear epithelial (NHMEE) cells using a double perfusion chamber, which enabled us to analyze membrane-specific transporter activities. NKCC activities were estimated by the $pH_i$ reduction due to bumetanide-sensitive intracellular uptake of $NH_4^+$. In addition, transepithelial fluid transport was measured with or without bumetanide-treatments. NKCC activities were observed in the basolateral membrane, but not in the luminal membrane of NHMEE cells. Correspondingly, immunoblotting of membrane-cultured NHMEE cells revealed the expression of NKCC1 on the basolateral membrane. Interestingly, IL-1ß treatments augmented the basolateral NKCC activities and increased NKCC1 expression. In addition, IL-1ß stimulated bumetanide-sensitive fluid transport in NHMEE cells. Furthermore, NKCC1 expression was increased in middle ear cells from the patients with OME when compared to samples from control individuals. The above results provide comprehensive evidence that the inflammatory cytokine IL-1β upregulates NKCC1 in the middle ear epithelial cells, which would be one of the important underlying mechanisms of fluid overcollection in patients with OME.

402

### Rapid Posttranslational Regulation of the Mouse Zinc Transporters ZIP4 and ZIP5 following Zinc Repletion

B. P. Weaver, G. K. Andrews; Biochemistry, KUMC, Kansas City, KS

ZIP4 and ZIP5 belong to the ZIP (Zrt-Irt-like proteins, SLC39A) family of metal ion transporters. Mutations in human ZIP4 cause acrodermatitis enteropathica which reflects an inability to absorb adequate zinc from the diet. ZIP5 is a close paralog to ZIP4. We have shown that both transporters undergo differential trafficking in response to dietary zinc depletion. To further examine the mechanism of regulation for ZIP4 and ZIP5, we analyzed proximal intestine and pancreas from weanling mice fed a zinc deficient (ZnD) or zinc adequate (ZnA) diet for 10 days followed by a gavage of zinc (or saline control). Tissues were collected for up to 6h after gavage (n = 4 for each group). Intestinal MT-I mRNA was upregulated after the zinc gavage. In contrast, intestinal ZIP4 mRNA was induced during ZnD but remained unchanged after zinc gavage and ZIP5 mRNA was unaltered by zinc. In mice fed a ZnA diet, ZIP4 protein was rare and was not detected on the apical membrane of enterocytes, whereas ZIP5 protein was detected on basolateral membranes of enterocytes and pancreatic acinar cells. During ZnD, ZIP4 protein accumulated at the apical membrane of enterocytes while ZIP5 was removed from the enterocytes and acinar cell membranes. Within 4h of a zinc gavage, ZIP4 protein was internalized and rapidly degraded in enterocytes whereas ZIP5 protein re-accumulated on the basolateral membranes of enterocytes and acinar cells. These effects were protein-specific since other apical and basolateral membrane proteins were unaffected by zinc and these effects were cell-specific since ZIP4 protein abundance in beta-cells was apparently unaffected by zinc.

403

**Characterizing Membrane Potential Changes during Ascidian Sperm Activation**

R. McNulty, R. A. Koch; Biological Science, CSU Fullerton, Fullerton, CA

Contact, both direct and indirect, between sperm and egg initiate cell signaling pathways that trigger internal responses. In the sea squirt sperm cells, these responses include increased swimming activity and mitochondrial translocation, an actin:myosin-dependent process that allows the sperm to penetrate the egg. Our laboratory has studied the latter response in *Ascidia ceratodes* and shown that calcium influx via penfluridol-sensitive channels is necessary for mitochondrial translocation. Thus, we have investigated the role of membrane potential change in sperm activation. We used the voltage-sensitive dye, DiS-C(3)5, to measure changes in sperm membrane potential in batches of sperm. The contribution of the mitochondrial potential was eliminated with FCCP. The artificial stimulus of high external pH induced a rapid hyperpolarization with a subsequent slow return to near resting potential. Experiments that varied external potassium ion concentrations ($[K^+]_o$) support a significant role for $K^+$ efflux in hyperpolarization. Reversal potential determined from this data predict an internal $[K^+]$ of 105mM. Stepwise addition of $K^+$, in presence of valinomycin, allowed the determination of a resting membrane potential of -40mV and a $K^+$ equilibrium potential of -62mV. Since high pH leads to hyperpolarizing membrane potentials around -250mV, it is likely the hyperpolarization results from multiple components. The potential role of $Cl^-$ influx in pH-induced hyperpolarization was also investigated by ion substitution with sodium gluconate. Decreasing $[Cl^-]_o$ decreased amplitude of hyperpolarization, until 100mM. The hyperpolarization was blocked with furosemide, supporting the involvement of inward rectifying K channels or chloride-permeable GABA-A ligand-gated channels. Hyperpolarization was also blocked with penfluridol and pimozide, supporting the idea calcium influx via T-type $Ca^{2+}$ channels precedes $K^+$-dependent hyperpolarization. We conclude pH-induced hyperpolarization depends on $K^+$ efflux and $Cl^-$ influx. Studies are under way to classify channels responsible for hyperpolarization and their relationship to calcium influx.

404

**Expression of $B^0AT1$ and LAT2 Amino Acid Transporters in Spontaneous Hypertensive Rat on High Salt Intake**

M. Pinho, P. Soares-da-Silva; Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal

The present study evaluated the activity of renal dopaminergic system and the expression of LAT2, 4F2hc and $B^0AT1$ in the renal cortex of the SHR and the normotensive Wistar-Kyoto rats (WKY), during normal (NS) and high (HS) salt intake. At 12 weeks of age, WKY failed to respond to HS with increases in the urinary dopamine excretion, while HS intake in SHR produced a marked increase in the urinary excretion of dopamine. L-DOPA plasma levels in SHR during NS and HS intake were similar to those in WKY. RT-PCR quantification analysis revealed a decreased expression of LAT2 mRNA in WKY and SHR on HS intake. At 12 weeks of age, LAT2/4F2hc ratios in SHR on HS intake were significantly lower (18 % reduction) than in SHR fed a NS diet, which was not the case in WKY. This suggested that a $Na^+$ dependent transport system may be promoting the L-DOPA uptake, this being most prominent in the SHR. Evaluation of the expression of $Na^+$ dependent $B^0AT1$, showed a significant decrease in both 4 and 12 week old WKY and in 4 week old SHR, fed HS diet. By contrast, HS intake produced a significant increase (~ 45% augment) in $B^0AT1$ mRNA of SHR of 12 weeks of age. It is concluded that increased renal dopamine production and excretion in SHR might be related to enhanced ability to take up L-DOPA at the kidney level. During HS, $Na^+$ dependent $B^0AT1$ is overexpressed in SHR kidney, probably contributing to the enhanced L-DOPA uptake, observed in the hypertensive phase. Differences in regulation of renal $B^0AT1$ and LAT2 in SHR versus WKY may have to do with differences concerning sodium handling. Supported by grant POCTI/SAU-OBS/57916/2004

405

**Cloning and Expression of Rat Neutral Amino Acid Transporter $B^0AT1$ (SLC6A19) in Renal Tissues**

M. Pinho, P. Soares-da-Silva; Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal

$B^0AT1$ is a novel member of the $Na^+$ dependent neurotransmitter transporter family (SLC6), and a candidate gene for the Hartnup disorder. Here, it is reported the cloning and molecular characterization of neutral amino acid transporter $B^0AT1$ in the rat kidney. A homology search was performed using BLASTN 2.2.5 (NCBI) and *Mus musculus* $B^0AT1$ sequence (XM_127449) against the non-redundant and EST databases. Three Rattus norvegicus putative $B^0AT1$ sequences were found on the EST database. The most significant being CK474548 with 834 nucleotides and CK473544 with 790 nucleotides, both belong to a cDNA library, which RNA was obtained from rat kidney. Identity of 90% and 93% were found between the mouse $B^0AT1$ and rat CK474548 and CK473544 sequences, respectively. Sets of primers were designed based on the alignments between known sequences. PCR and 5' RACE were performed using cDNA obtained from total RNA from kidney cortex of WKY rat. Fragments obtained were cloned and sequencing. The sequence obtained for rattus norvegicus $B^0AT1$ transcript has 2360 bp, and alignments with human and mouse homologues revealed identities of 84.4% and 94.3%, respectively. The expression of $B^0AT1$ transcript was also examined by RT-PCR using specific primers in SHR and WKY immortalized renal proximal tubular epithelial cells, both cell lines amplified the expected product of 1026 bp. Supported by grant POCTI/SAU-OBS/57916/2004.

406

**Functional Characterization of $Na^+$-$HCO_3^-$ Cotransporter in WKY and SHR Proximal Tubular Epithelial Cells**

R. Pedrosa, N. Gonçalves, P. Soares-da-Silva; Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal

The present study was designed to evaluate the presence and characterize the functional proprieties of the $Na^+/HCO_3^-$ cotransporter in immortalized proximal tubular epithelial cells from spontaneous hypertensive rat (SHR) and their normotensive controls (Wistar Kyoto rats; WKY). The $Na^+$-$HCO_3^-$ cotransporter activity was assayed as the initial rate of the $Na^+$-dependent $pH_i$ recovery ($dpH_i/dt$, pH/s) in a $HCO_3^-$ containing solution after an acid load induced by $NH_4^+$ (20 mM) in the absence of $Na^+$. After intracellular acidification and in the presence of amiloride (1 mM), the addition of $Na^+$ (115 mM) in the absence of $Cl^-$ resulted in rapid pHi recovery in both WKY (0.00227 ± 0.00019 pH units/s) and SHR (0.00168 ± 0.00012 pH units/s) cells. This recovery was clearly $Na^+$ and $HCO_3^-$ dependent in both WKY and SHR cells. DIDS (4,4'-diisothiocyanatodihydrostilbene-2,2'-disulphonic acid; 300 µM) markedly inhibited the $Na^+$ and $HCO_3^-$-dependent pHi recovery phase. Taken together these results suggest that WKY cells and SHR cells are endowed with a $Na^+$-$HCO_3^-$ cotransporter. Chronic acidosis induced by 24 h treatment with $NH_4^+$ (20 mM) increased $Na^+$-$HCO_3^-$ cotransport activity that was greater in SHR cells (79.8 ± 13.3 % increase) than in WKY cells (37.7 ± 2.9 % increase), with no changes in intracellular pH and cell viability (93.8 ± 2.4 and 99.4 ± 1.4 % of live cells; WKY and SHR). Treatment with acetozalamide (300 µM) for 24 h

did not change the $Na^+$-$HCO_3^-$ activity in WKY and SHR cells. It is concluded that the activity of the $Na^+$-$HCO_3^-$ cotransporter in SHR cells was lower than that in WKY, but the former responds with an enhanced response to acidosis-induced stimulation of $Na^+$-$HCO_3^-$ activity. Supported by grant POCTI/SAU-FCF/59207/2004.

407

**Enhanced Sensitivity to Angiotensin II-induced Stimulation of $Cl^-/HCO_3^-$ Exchanger in Immortalized SHR Proximal Tubular Epithelial Cells**

R. Pedrosa, P. Soares-da-Silva; Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal

We have recently reported an increased activity of the apical $Cl^-/HCO_3^-$ exchanger (SLC26A6) in immortalized proximal tubular epithelial cells from spontaneous hypertensive rat (SHR) when compared with their normotensive controls (Wistar Kyoto rats; WKY). The present study evaluated the expression and function of SLC26A6 after angiotensin II (Ang II) in immortalized proximal tubular epithelial cells from SHR and WKY. The expression of SLC26A6 in SHR cells was 7 fold (704.0±115.2 %) that in WKY cells. The exposure of cells to Ang II for 25 min induced an increase in SLC26A6 activity with an $EC_{50}$ value of 0.10 (0.05, 0.22) nM and 11.85 (7.63, 18.39) nM in SHR and WKY cells, respectively. An equipotent stimulatory effect induced by 1 nM Ang II (178.4±14.4 % stimulation) in SHR cells and 30 nM Ang II (175.7 ± 25.0 % stimulation) upon SLC26A6 was completely blocked by losartan (100 nM) in both cell types. By contrast, the effect of Ang II was unaffected by the specific AT2 antagonist PD 123,319 (100 nM). A short treatment (5 min) with Ang II (10 nM) induced an increase in SLC26A6 activity in SHR cells, but not in WKY cells. These effects were antagonized by losartan (100 nM) but not by PD 123,319 (100 nM). It is concluded that SHR cells have an increased activity of SLC26A6, as a result of the higher expression of this transporter. SHR cells also have an enhanced sensitivity to Ang II-induced stimulation of the $Cl^-/HCO_3^-$ exchanger activity through the AT1 receptor. Supported by grant POCTI/SAU-FCF/59207/2004.

408

**KCl Cotransport is Essential for Insulin-like Growth Factor 1-mediated Invasiveness and Proliferation of Breast Cancer Cells**

M. Shen; Department of Pharmacology, National Cheng Kung University, College of Medicine, Taiwan, Taiwan Republic of China

Electroneutral K-Cl cotransport (KCC), via the regulation of ionic and osmotic homeostasis, has begun to emerge of its important roles in cancer development, tumor invasion, and possibly metastasis. We test the hypothesis that insulin-like growth factor 1 (IGF-1) may promote breast cancer development and progression through its action on KCC. IGF-1 enhanced KCC-dependent cell volume regulation. IGF-1 treatment triggered phosphatidylinositol 3-kinase and mitogen-activated protein kinase cascades which were differentially required for IGF-1-stimulated biosynthesis of KCC. IGF-1 dose-dependently stimulated cellular invasiveness and proliferation, which were almost abolished by KCC inhibitor. The invasiveness and proliferation of KCC dominant-negative mutant cells were insensitive to IGF-1 stimulation. KCC mutation significantly decreased xenograft tumor size in SCID mice although the phosphorylation of IGF-1 receptor was not changed. In addition to colocalization, the expression level of IGF-1 and KCC in the same surgical specimen showed a good linear correlation, suggesting autocrine or paracrine IGF-1 stimulation of KCC production *in vivo*. Among patients with early-stage node-negative breast cancer, disease-free and overall survival curves were significantly different based on IGF-1 and KCC expression. Thus, we conclude that KCC activation by IGF-1 plays an important role in IGF-1 signaling to promote growth and spread of breast cancer cells.

409

**Interaction of Cation Channel TRPV4 and Actin Cytoskeleton**

D. Becker, R. Ramadass, C. Blase, M. Jendrach, J. Bereiter-Hahn; Kinematic Cell Research Group, Biocenter Frankfurt University, Frankfurt, Germany

The non-selective cation channel TRPV4 is involved in many different physiological processes like osmoregulation and temperature sensing. We investigated activation mechanism of TRPV4 in response to hypotonic stress using $Ca^{2+}$ imaging and fluorescence lifetime imaging microscopy (FLIM). Under hypotonic conditions TRPV4 is activated and permeable to $Ca^{2+}$ ions, which leads to a transient $[Ca^{2+}]_i$ increase. The hypotonicity induced $[Ca^{2+}]_i$ increase was abolished in cells treated with F-actin disrupting reagents. Confocal laser scanning microscopy (CLSM) of phalloidin stained and GFP-TRPV4 transfected cells showed co-localisation in plasma membrane. To evaluate interaction, fluorescence resonance energy transfer (FRET) studies, using picosecond FLIM were performed. CFP-TRPV4 and YFP-actin double transfected CHO cells were observed with a fluorescence decay microscope, calibrated with a full width at half maximum (FWHM) of 75 ps for the temporal resolution. CFP-TRPV4 was excited at 440nm and fluorescence decays, both spectrally and spatially resolved, were obtained. In CFP-TRPV4 and YFP-actin double transfected cells, the lifetime of the fluorescence decay obtained at 470-480nm was around 40% reduced in the shorter lifetime, compared to CFP-TRPV4 single transfected cells. Also, in the case of spectrally resolved fluorescence decays an emission peak corresponding to YFP fluorescence at 530nm was seen. Taken together, these results indicate close proximity and interaction of TRPV4 and actin cytoskeleton.

410

**Nedd4-2-mediated Ubiquitination and Degradation of ENaC**

R. Zhou, P. M. Snyder; Internal Medicine, Physiology and Biophysics, University of Iowa, Iowa City, IA

The epithelial Na+ channel (ENaC) is an amiloride-sensitive ion channel composed of three subunits (alpha, beta, and gamma ENaC) that plays a critical role in Na+ homeostasis and blood pressure control. ENaC is regulated by Nedd4-2, an E3 ubiquitin-protein ligase. Here we tested the hypothesis that Nedd4-2 inhibits ENaC by ubiquitination of ENaC subunits. We found that Nedd4-2 induced ubiquitination of all three subunits either in the total protein pool or at cell surface. Nedd4-2 binds to cell surface ENaC followed by ubiquitination, which had a selective effect on the pool of ENaC at the cell surface; using a biotinylation assay, we found that Nedd4-2 produced a dose-dependent decrease in ENaC at the cell surface, but had no effect on the cytoplasmic pool of ENaC. Nedd4-2 decreased ENaC surface expression by increasing the rate of degradation of cell surface ENaC. Mutation of a cysteine in the Nedd4-2 HECT domain required for ubiquitin ligase activity abolished the ability of Nedd4-2 to decrease ENaC surface expression and catalyze ENaC ubiquitination. The Nedd4-2-mediated decrease in ENaC surface expression and increase in ENaC ubiquitination were also abolished or attenuated by mutations in ENaC PY motifs (that cause Liddle's syndrome) or Nedd4-2 WW domains which mediate binding to ENaC. We found that SGK and PKA blunted the ability of Nedd4-2 to decrease ENaC surface expression. Conversely,

mutation of the Nedd4-2 phosphorylation sites increased ENaC degradation. Together the data suggest that Nedd4-2 modulates epithelial Na+ transport by ubiquitination of ENaC, which selectively targets ENaC at the cell surface for degradation. Nedd4-2-mediated degradation is regulated by SGK and PKA, down-stream mediators of the aldosterone and vasopressin pathways. Defects in this regulation are responsible for Liddle's syndrome, an inherited form of hypertension, and could contribute to the pathogenesis of essential hypertension.

411

**Soluble Interaction Domains in hERG1a/1b Heteromeric Channels**

P. Phartiyal, E. M. C. Jones, G. A. Robertson; Physiology, University of Wisconsin-Madison, Madison, WI

Channels producing $I_{Kr}$ currents in the human heart are composed of two subunit species encoded by alternate transcripts of the *human Ether-a-go-go-Related Gene* (*hERG*), but the mechanisms regulating channel assembly and composition are unknown. In heterologous expression systems, the two subunits readily assemble to produce heteromeric channels with robust currents. When expressed in isolation, hERG1a also produces large currents but hERG1b produces little or no current. hERG1a and hERG1b subunits are identical except for the N termini, which are largely unique. Previous studies have shown subunit assembly of Kv channels progresses cotranslationally, with specificity determined by the association of nascent N termini emerging from the ribosome. We tested the hypothesis that hERG 1a and 1b N termini interact to promote heteromeric assembly of hERG subunits. We also tested the hypothesis that hERG1b subunits fail to assemble homotypically owing to electrostatic repulsion between N termini bearing a net positive charge. We found the hERG1a and 1b N termini have a direct and dose-dependent interaction as assayed by GST fusion protein interactions. In addition to the N-N interactions, association between the N and C termini was also observed to be robust and dose-dependent. However, 1a and 1b subunits with nearly complete C-terminal truncations coexpressed in HEK-293 cells were able to assemble, indicating heteromerization does not depend on the presence of the C terminus. Surprisingly, 1b subunits were also shown to interact homotypically as assayed by co-immunoprecipitation experiments, indicating that low 1b current density is not attributable to N-terminal repulsion and failed assembly. Results from this study identify key soluble interaction domains involved in hERG heteromerization and present the first step toward dissecting the assembly process of this important heteromeric potassium channel. Supported by NIH grant HL68868.

412

**Endothelin-1 Regulation of Caveolar $Ca_v1.2$ Channel Function Involves a Multiprotein Signaling Complex Including Non-Receptor Tyrosin Kinases in Cardiac Myocytes**

R. C. Balijepalli,[1] J. D. Foell,[1] K. Y. Chung,[2] J. L. Walker,[2] T. J. Kamp[1]; [1]Medicine, University of Wisconsin, Madison, Madison, WI, [2]Physiology, University of Wisconsin, Madison, Madison, WI

Endothelin-1 regulation of cardiac function is in part due to the stimulation of influx of $Ca^{2+}$ through L-type $Ca^{2+}$ channels in ventricular cardiomyocytes. Previously we have demonstrated the localization of L-type $Ca^{2+}$ ($Ca_v1.2$) channels and endothelin-A receptors ($ET_AR$) in caveolar microdomains in ventricular myocytes. However, the critical intermediate proteins in the signaling cascade linking $ET_AR$ and regulation of $Ca_v1.2$ channels have not been described in detail. We utilized immunoprecipitation (IP) and proteomics approaches to characterize the signaling complexes associated with $ET_AR$ and $Ca_v1.2$ channels in caveolae. $Ca_v1.2$, caveolin-3 and $ET_A$-receptor were separately immunoprecipitated from mouse ventricular homogenates using respective specific antibodies, and associated proteins were resolved on SDS-PAGE gels. Trypsin and Glu-C digested peptide masses were subjected to MALDI-TOF mass-spectrometry and Swiss-Prot database analysis. Proteomic analysis of the three IPs revealed significant overlap of constituent proteins including membrane receptor proteins, ion channels, cytoskeletal proteins, trafficking proteins and kinases such as non-receptor tyrosine kinases including Pyk2 and c-Src. To specifically investigate the role of non-receptor tyrosine kinases in the functional regulation of $Ca_v1.2$ channels, we performed whole-cell patch clamp experiments in neonatal mouse ventricular myocytes. ET-1 (100 nmol/L) stimulation of ventricular myocytes significantly increased $I_{Ca,L}$ by 59±16% (n=3, $p<0.05$), and this ET-1 effect was abolished by pretreatment of cells with tyrosine kinase inhibitors genistein (50µM) and c-src inhibitor PP2 (50µM). In addition, ET-1 induced Tyr-phosphorylation of the $Ca_v1.2$ channel, which was blocked by pretreatment with genistein or PP2. We conclude that non-receptor tyrosine kinases as part of a macromolecular signaling complex are essential in mediating the ET-1 regulation of $I_{Ca,L}$. Understanding the details of this signaling pathway may provide new insights into the regulation of heart function in health and disease.

413

**hERG-RUFY2 Interactions in the Endocytic Pathway**

S. A. Delfosse, E. C. Roti Roti, E. M. C. Jones, G. A. Robertson; Dept. of Physiology, University of Wisconsin-Madison, Madison, WI

Cardiac excitability is determined not only by the types of ion channels present in the surface membrane but also by their relative densities. One mechanism important in regulating surface channel density is endocytosis, a first step in both degradation pathways and recycling. In searching for proteins involved in hERG channel trafficking, we identified RUFY2 in a yeast two-hybrid screen of a human heart library using the hERG C terminus as bait. RUFY2 is homologous to the RUFY1 rab4-interacting protein implicated in the recycling endosomal pathway. Antibodies raised specifically against RUFY2 identified a 75 kD protein in human heart lysate. Subcellular fractionation of HEK-293 revealed colocalization of hERG with RUFY2 in endosomal fractions verified by the presence of EEA1, an early endosomal marker. hERG and RUFY2 also had overlapping distributions in sub-plasma membrane compartments as demonstrated by immunocytochemistry. These findings are consistent with a role for RUFY2 in hERG trafficking, possibly by regulating surface expression via channel turnover and/or recycling. Supported by NIH grant HL68868.

414

**GM130 Sorts hERG Channels Based on Subunit Composition**

E. C. Roti Roti, E. M. C. Jones, G. A. Robertson; Dept. of Physiology, University of Wisconsin-Madison, Madison, WI

*hERG*, the *human Ether-a-go-go-Related Gene*, encodes a voltage-gated potassium channel expressed as two isoforms, 1a and 1b, which coassemble to form heteromeric channels underlying cardiac $I_{Kr}$ (Jones et al JBC 279:44690, 2004). $I_{Kr}$ can be disrupted by inherited mutations, giving rise to long QT syndrome (LQTS) and potentially fatal cardiac arrhythmias. Most mutations characterized to date disrupt trafficking of hERG channels, but the underlying molecular mechanisms are poorly understood. We are characterizing hERG-interacting proteins in an effort to elucidate steps in the trafficking pathway that regulate surface expression and to identify potential targets for disease. We previously showed that

GM130, a *cis*-Golgi matrix protein involved in vesicular transport between the ER and Golgi, also interacts with hERG as a cargo molecule (Roti Roti et al JBC 277:47779, 2002). We tested the hypothesis that GM130 is responsible for sorting 1a homomers and 1a/1b heteromers, which traffic effectively to the plasma membrane, from 1b homomers, which do not. In binding assays, GM130 showed a strongly preferential interaction with hERG 1b homomers. Consistent with the binding data, hERG 1b homomers accumulated in Golgi compartments. These data suggest GM130 sorts hERG oligomers for surface expression based on subunit composition. Supported by NIH grant HL68868 (GAR) and NIH NRSA T32 HL07936 from the University of Wisconsin-Madison Cardiovascular Research Center (ECRR).

415

**Direct Phosphorylation of a Voltage-Gated Potassium Channel by PKA Modulates its Expression on the Cell Surface**

E. C. Connors, A. D. Morielli; Pharmacology, University of Vermont, Burlington, VT

Trafficking of ion channels to and from the cell surface is crucial for regulation of membrane potential and is often controlled by direct phosphorylation of the channel protein. The voltage-gated potassium channel Kv1.2 has been shown to be negatively regulated by tyrosine phosphorylation and subsequent trafficking away from the plasma membrane through endocytosis. In this study, we investigate mechanisms for positive regulation of the channel through phosphorylation by protein kinase A (PKA). Using flow cytometry to quantify the level of Kv1.2 on the cell surface, we found that a 10 minute application of 10μM forskolin, increased the amount of Kv1.2 on the cell surface by 54.01 +/- 0.06% (p=8E-05; n=53). Inhibitors of PKA, KT5720 (5μM; p=0.22; n=20) and PKI (p=0.20; n=3), but not a mutant form of PKI that does not bind the catalytic subunit of PKA (p=0.004; n=3), blocked the response. Previous electrophysiology data had shown that a mutation within the N-terminus of the channel, threonine 46 to valine (T46V), blocked channel potentiation via PKA. T46V channels are also resistant to forskolin-induced increases of Kv1.2 levels at the cell surface (p=0.488; n=9). This data indicates that PKA mediated potentiation of the Kv1.2 ionic current occurs in part by increasing the quantity of Kv1.2 channels expressed at the surface of the cell.

416

**Activation of 5-$HT_{2B}$ Receptor Stimulates $Ca^{2+}$ Influx through TRPC6 Channels in Pelvic Autonomic Transmission**

S. Cha, I. Kong; Department of Physiology, Yonsei Univ. College of Medicine, Wonju, Republic of Korea

Pelvic ganglia provide autonomic innervations to the various pelvic organs such as colon, bladder and penis. Several studies have shown that 5-HT is present in some neurons of the pelvic ganglia, depolarizes neurons and inhibits ganglion transmission. In pelvic ganglia, however, mechanisms underlying actions of 5-HT remain unknown. We examined the role of 5-$HT_2$ receptor in pelvic autonomic transmission using patch-clamp and fluorescence $Ca^{2+}$ measurement techniques. In both sympathetic and parasympathetic neurons, 5-HT (10 μM) itself and BW723C86 (10 μM), a 5-$HT_{2B}$ agonist, increased $[Ca^{2+}]_i$ , which were prevented by SB204741 (10 μM), a 5-$HT_{2B}$ antagonist. This 5-$HT_{2B}$-mediated $[Ca^{2+}]_i$ increase was blocked by removal of extracellular $Ca^{2+}$, by nonselective cation channel blockers, $Gd^{3+}$ (10 μM) or $La^{3+}$ (100 μM), and by U73122 (1 μM), a PLC inhibitor with no effect of U73343 (1 μM), an inactive analogue of PLC. Under a voltage-clamp condition, BW723C86 (100 μM) also caused an inward current in a $La^{3+}$-sensitive manner. Interestingly, OAG (100 μM), a membrane permeable DAG analogue, induced $Ca^{2+}$ influx. RT-PCR analysis confirmed TRPC1 and TRPC6 expressed in MPG neurons. Taken together, these data suggest that $Ca^{2+}$ influx occurs through TRPC6 channels when activation of 5-$HT_{2B}$ receptors stimulates the $G_q$-PLC-DAG pathway.

417

**Membrane Targeting of Voltage-Gated Sodium Channel $Na_v1.6$**

A. Lee, A. L. Goldin; Microbiology & Molecular Genetics, University of California, Irvine, Irvine, CA

Voltage-gated sodium channels are membrane proteins that initiate action potentials in neurons following membrane depolarization. Members of this family show differential distribution at the subcellular level. The mechanisms underlying the targeting of these isoforms are not understood. However, their specificity is important because the isoforms can change the excitability of the membrane due to differences in their electrogenic properties. In this study, we examined the $Na_v1.6$ structure for a localization determinant. $Na_v1.6$ is the major isoform found at nodes of Ranvier. Using a fluorescent tag, we showed that $Na_v1.6$ segregated to the basolateral membrane domain of a polarized renal epithelial (MDCK) cell. In contrast, $Na_v1.2$--another major isoform found in the axon--appeared in the apical membrane domain. Chimeric constructs helped to narrow down the $Na_v1.6$ targeting signal to a 200 amino acid sequence region. Mutations within this region prevented $Na_v1.6$ from accumulating in the basolateral membrane. These results suggest that the $Na_v1.6$ channel carries a membrane-sorting signal within this sequence region and raise the probability that the region also plays a role in the nodal targeting of the $Na_v1.6$ channel in the neuron. The targeting effect of this sequence in neurons is currently under investigation.

418

**Post-transcriptional Regulation of Multidrug Resistant Efflux Pumps in Bacteria**

K. A. Gibbs, J. A. Theriot; Biochemistry Department, Stanford University, Stanford, CA

Resistance to toxins is crucial for the survival of all cells. In addition to toxin-specific mechanisms of resistance attained through transcriptional and translational regulation of specific enzymes, both eukaryotes and prokaryotes employ general integral membrane pumps to efflux small metabolites from the inside of the cell. Bacteria, in addition to fungi and other eukaryotes, encode membrane pumps that are able to export a range of antibiotics from different classes. These multidrug resistant (MDR) efflux pumps are increasingly found to be elevated in clinically isolated drug-resistant strains of pathogenic bacteria including *Escherichia coli* and *Pseudomonas aeruginosa*. We have identified a novel mechanism by which these efflux pumps are able to provide increased survival on acute exposure to antibiotics. We have engineered a plasmid encoding the MDR pump, MdfA, fused to Green Fluorescent Protein (GFP) under the control of the *lac* promoter. Wild-type MdfA is present on the chromosome. Cells carrying the MdfA-GFP transgene are able to continue to proliferate in concentrations of tetracycline and ampicillin bacteriostatic to wild-type *E. coli*. Strikingly, these cells express MdfA-GFP when exposed to tetracycline and ampicillin even in the absence of IPTG induction. In the absence of antibiotics, these same uninduced cells exhibit no fluorescence. Since the MdfA-GFP transgene is under the control of the *lac* promoter, it is unlikely that antibiotic exposure is regulating protein expression at the level of transcription. We propose that small metabolites, such as antibiotics, are able to regulate the expression or stability of MDR efflux pumps by directly stabilizing the protein. This effect may contribute to the

increased survival of bacteria, and perhaps eukaryotic cells, when exposed to antibiotics and other toxic small metabolites.

## Membrane Fusion (419-427)

419

### Efficient SNARE Mediated Fusion Requires Asymetric Phopholipid Distribution

J. A. Vicogne, D. Vollenweider, J. R. Smith, J. E. Pessin; Pharmacology, SUNY Stony Brook, Stony Brook, NY

GLUT4 transport vesicle fusion with the plasma membrane requires the plasma membrane t-SNAREs (Syntaxin 4 and SNAP23) and the vesicle v-SNARE (VAMP2). Although extensive studies have demonstrated that in vitro the neuronal t-SNAREs Syntaxin1/SNAP25 and the v-SNARE VAMP2 can function as the “minimal machinery” for the membrane fusion reaction, there have been no studies examining the functional properties of the GLUT4 SNARE proteins. Using proteoliposomes containing Syntaxin 4/SNAP23 and proteoliposomes containing VAMP2, we demonstrated that these SNAREs are capable of driving vesicle fusion with a similar rate and extent as the neuronal SNAREs. Moreover, reconstitution of the t-SNARE proteoliposomes with phosphatidic acid (PA) markedly enhanced the rate of the fusion reaction whereas reconstitution of the VAMP2 proteoliposomes with phosphatidic acid markedly reduced the rate of vesicle fusion. In contrast, reconstitution of the VAMP2 proteoliposomes with phosphatidylinositol-4,5-P2 (PIP2) increased vesicle fusion whereas PIP2 in the Syntaxin4/SNAP23 proteoliposomes inhibited vesicle fusion. Since PA potentiates negative membrane curvature and PIP2 supports positive membrane curvature, these data suggests that there is an inherent asymmetry in SNARE-mediated fusion reaction. We conclude that the rate of fusion can be regulated by membrane lipid composition probably by facilitating the action of SNAREs to open the fusion pore.

420

### SNARE Trans-Membrane Domains Regulate the Progression of Hemifused Yeast Vacuoles to Full Fusion

Y. Jun, W. Wickner; Biochemistry, Dartmouth College, Hanover, NH

While influenza HA-mediated membrane fusion fusion proceeds through a hemifusion intermediate, it remains yet to be determined whether Rab- and SNARE-mediated intracellular membrane fusion entails a hemifusion state. During the docking which preceeds yeast vacuole fusion, a ring of fusion factors surround disc-like apposed "boundary membrane" domains from each vacuole. As for lipid-anchored HA protein, lipid-anchored SNAREs support trans-SNARE pairing and lipid mixing but not content mixing. The full fusion of vacuoles with lipid-anchored SNARE can be achieved by modifying the curvature of inner membrane leaflet with membrane-permeable amphiphiles. These results suggest that Rab- and SNARE-mediated membrane fusion proceeds through a hemifusion intermediate and that the transmembrane domain of SNARE is important for progression to full fusion. We propose that the boundary membrane is a novel vertex ring hemifusion diaphragm of two bilayer membranes. This model explains the abundance of vacuole proteins and exterior leaflet lipids in the boundary membrane, as well as the generation of lumenal vesicles upon membrane fusion.

421

### A Dominant Negative Version of Vps45p Reveals Two Modes of Binding to its Cognate SNAREs

L. N. Carpp,[1] L. F. Ciufo,[2] S. G. Shanks,[1] A. Boyd,[2] N. J. Bryant[1]; [1]Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, United Kingdom, [2]School of Biomedical Sciences, The Physiological Laboratory, University of Liverpool, Liverpool, United Kingdom

Sec1/Munc18 (SM) proteins have been shown to be essential in SNARE mediated membrane fusion events. Their exact mode of action remains elusive. Crystal structures of two SM proteins with their cognate Syntaxins have been solved. Munc18a and Sly1p (involved in synaptic transmission and ER-to-Golgi transport, respectively) are both arch shaped molecules with a central cavity. Syntaxin1a sits in the central cavity of Munc18a and makes multiple contacts with the SM protein. The binding of Sly1p to Sed5p (Syntaxin5) is quite distinct from this and involves a hydrophobic pocket on the outer face of the SM protein. This binding is facilitated by a short peptide at the extreme N-terminus of the Syntaxin. We have previously demonstrated that the SM protein Vps45p binds to both its monomeric Syntaxin, Tlg2p, and also to Tlg2p-containing *cis*-SNARE complexes. We have now isolated a dominant-negative version of Vps45p that has enabled us to obtain data supporting a model in which Vps45p binds to Tlg2p, not only via a mode analogous to that described for Sly1p/Sed5p, but also by a distinct mode. Implications of this model for SM protein function will be discussed

422

### Purification, Activity Assay, and Phosphoinositide Binding of the HOPS/Class C Vps Complex

C. Stroupe; Dept. of Biochemistry, Dartmouth Medical School, Hanover, NH

The mechanisms by which Rab GTPase activation is coupled to SNARE complex assembly are unclear. The HOPS/Class C Vps complex is a nucleotide exchange factor and an effector for the vacuolar Rab protein Ypt7p, and is also required for formation of vacuolar SNARE complexes. We have developed a purification method and an activity assay for the HOPS complex. Using pure, active HOPS, we have found that the HOPS complex is required for vacuole tethering and for catalysts of vacuole docking to complete their functions during vacuole fusion. We have also shown that the HOPS complex binds specifically and directly to phosphoinositides. Phosphoinositide binding is required for vacuolar association of the HOPS complex and for its localization in the same membrane microdomains on docked vacuoles as Ypt7p and SNARE proteins. Concentration of the HOPS complex in these microdomains may be a key factor for its activity in linking active Ypt7p to SNARE complex assembly.

423

### The Lgl Proteins Interact with the Exocyst Complex and Regulate Exocytosis and Cell Polarization

X. Zhang, P. Wang, W. Guo; Department of Biology, University of Pennsylvania, Philadelphia, PA

The exocyst is a multiprotein complex essential for tethering post-Golgi secretory vesicles at specific domains of the plasma membrane for exocytosis. The exocyst functions upstream of the SNAREs, and regulates SNARE assembly and subsequent membrane fusion. However, how the exocyst interacts and regulates the SNAREs is unclear. Here we report that Exo84p, a component of the exocyst complex, directly interacts with the Lgl proteins, Sro7p and Sro77p, which also bind to the t-SNAREs. Disruption of the interaction between the exocyst and Sro7/77p results in

defects in post-Golgi secretion. Genetic analyses of an array of post-Golgi secretory mutants demonstrate a molecular pathway from the Rab and Rho GTPases, through the exocyst and Lgl, to the SNAREs. We also found that overexpression of Lgl and the t-SNARE protein not only improves exocytosis but also rescues the polarity defects in exocyst mutants. We propose that Lgl proteins play an important role in promoting the coupling between the vesicle tethering machinery (the exocyst) and the fusion machinery (the SNAREs). Although Lgl is broadly distributed in the cells, its localized interaction with the exocyst and kinetic activation are important for exocytosis and the establishment and re-enforcement of cell polarity. The Lgl was originally identified as a tumor suppressor that plays a critical role in epithelial cell polarization. This study not only helps us understand the molecular basis of exocytosis, but also sheds light on the mechanisms of Lgl function in cell polarity.

424

**Involvement of syntaxin 18, an Endoplasmic Reticulum (ER)-localized SNARE Protein, in ER-mediated Phagocytosis**

K. Hatsuzawa,[1,2] H. Hashimoto,[1,2] Y. Yokoya,[1,2] H. Hashimoto,[1,2] T. Tamura,[1,2] I. Wada[1,2]; [1]Dept Cell Sci./Inst Biomed Sci, Fukushima Medical University, Fukushima, Japan, [2]CREST/JST, Kawaguchi-Saitama, Japan

The endoplasmic reticulum (ER) is thought to be a major source of membrane production during phagocytic invagination. Consistent with this, recent reports show that fusion of the ER to phagosomes is responsible for antigen cross-presentation mediated by MHC class I molecules. However, the exact mechanisms used to regulate the fusion remain elusive. In this study, we investigated the involvement of various SNARE proteins in membrane fusion between the ER and plasma membranes. When phagosomes were isolated from murine macrophages, J774 cells, we found a significant enrichment of ER-localized SNARE proteins (syntaxin 18 (syx18), D12, and Sec22b) in the fraction. Similarly, J774 cells stably expressing SNARE proteins tagged to GFP variants showed the accumulation of fluorescence on the phagosomal membranes. To determine whether those SNARE proteins are required for phagocytosis, we generated the 293T cells stably expressing Fcγ receptor, in which phagocytosis occurs in an IgG-mediated manner, and then we examined the effects of overexpression of the dominant-negative mutants lacking transmembrane domains. Interestingly, the inhibition was observed only in the cells overexpressing the soluble forms of syx18 and D12, but not that of Sec22b. Furthermore, when the expression of syx18 was inhibited by siRNAs, the phagocytosis efficiency was selectively decreased. Finally, we found that J774 cells overexpressing syx18 showed enhanced ingestion of luminol-beads. Therefore, we conclude that syx18 is required for ER-mediated phagocytosis, presumably through regulating specific and direct fusion of the ER and plasma membranes.

425

**Cell Cycle Regulation of Snare-mediated Membrane Fusion**

X. Ding,[1,2] K. Jiang,[1,3] C. Fu,[1] T. Ward,[1] H. Kim,[4] X. Yao[1,3]; [1]Physiology, Morehouse School of Medicine, Atlanta, GA, [2]Beijing Univ. of Medicine, Beijing, China, [3]Univ. of Sci. & Tech. of China, Hefei, China, [4]Pharmacology, Univ. of Alabama, Birmingham, AL

Cytokinesis is an essential process that segregates cellular contents into daughter cells. Aurora-B, a mitotic protein kinase, plays important roles in metaphase chromosome alignment, chromosome segregation, and cytokinesis. It has been shown that successful completion of cytokinesis requires a SNARE-mediated membrane fusion event at the midbody, a cytoplasmic bridge that connects the two prospective daughter cells. However, the molecular mechanisms underlying such cell cycle-dependent membrane fusion remain elusive. Here we show Aurora B interacts and phosphorylates syntaxin 2 in vitro and in vivo. In addition, Aurora B is co-distributed with syntaxin 2 in the midbody between daughter cells. Mass spectrometric analyses of syntaxin 2 phosphorylated in vivo and in vitro have identified a conserved phosphorylation site in syntaxin 2 subfamily. Preventing Aurora B-mediated phosphorylation of syntaxin 2 inhibits the assembly of SNARE complex and causes failure of cytokinesis leading to the formation of binucleated cells. Moreover, this phosphorylation of syntaxin 2 is reversed in vitro by Cdc14A. Thus, phosphoregulation of the syntaxin 2 by Aurora B ensures that SANRE complex assembly occurs at the appropriate time in the cell cycle and maintains genomic stability.

426

**Fusing Partner Cells Require Mutual Expression of the Autonomous Cellular Fusogen EFF-1**

J. J. del Campo, J. H. Shinn, M. R. Schiller, C. J. Staber, R. A. Reenan, W. A. Mohler; Dept. of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT

Most cell fusion events in *Caenorhabditis elegans* require the phyletically distinct protein EFF-1 [1, 2, 3 ]. Inappropriate EFF-1 expression can also induce ectopic fusion fate in *C. elegans* tissues [2, 3]. We have tested the homotypic specificity and molecular autonomy of EFF-1 in driving cell fusion, and have begun to explore potential protein-interaction partners. First, we used genetic mosaic analysis in *C. elegans* to reveal a reciprocal requirement for EFF-1 expression in fusing cells. This result concurs with our observations of the dynamic localization of EFF-1::GFP in fusing embryonic cells [2]. Second, we tested the intrinsic sufficiency of EFF-1 by expression in a heterologous organism that does not possess an EFF-1 ortholog, showing that EFF-1 alone is sufficient to cause *Drosophila* neurons to undergo cell fusion. Therefore, EFF-1 appears to function independently, requiring simply that both partner cells express the protein in order to fuse. These properties make EFF-1 unique among known membrane fusogens, and may allow simple yet precise application of enforced cell fusion in experimental applications. Third, we have identified two potential 14-3-3-binding phosphopeptide motifs within the predicted cytoplasmic domain of EFF-1, and showed that they are required for cell fusion activity. These results inspire a model in which a combination of *cis* and *trans* interactions govern the highly specific targeting of EFF-1 to fusion-fated cell contacts in developing tissues. 1. Mohler, W.A., Shemer, G., del Campo, J.J., Valansi, C., Opoku-Serebuoh, E., Scranton, V., Assaf, N., White, J.G., and Podbilewicz, B. (2002). DevCell *2*, 355-362. 2. del Campo, J.J., Opoku-Serebuoh, E., Isaacson, A.B., Scranton, V.L., Tucker, M., Han, M., and Mohler, W.A. (2005). CurrBiol *15*, 413-423. 3. Shemer, G., Suissa, M., Kolotuev, I., Nguyen, K.C., Hall, D.H., and Podbilewicz, B. (2004). CurrBiol *14*, 1587-1591.

427

**Proteomic Analysis of Cell-cell Fusion during Mating in S cerevisiae**

X. Bai, S. Liu, J. A. McNew; Biochemistry and Cell Biology, Rice University, Houston, TX

Cell-cell membrane fusion is an essential yet elusive process in eukaryotic organisms. Little is known about the mechanism of cell-cell membrane fusion. We are attempting to identify the fusogens mediating plasma membrane fusion during the mating reaction in *Saccharomyces cerevisiae.* Our strategy assumes that the potential fusogens are induced in response to mating pheromone and transported through the traditional secretory

pathway. Post-Golgi secretory vesicles were accumulated in a temperature sensitive *sec6-4*$^{ts}$ strain treated with synthetic alpha mating factor. The accumulated vesicles were purified by differential centrifugation and density sedimentation. Peak vesicle fractions were identified by immunoblot analysis with an antibody directed against the post-Golgi vesicle SNARE Snc1/2p. Peak vesicle fractions from cell treated with alpha factor and control untreated cells were analyzed by two-dimensional polyacrylamide gel electrophoresis. Visual inspection revealed approximately 80 protein spots with subtle differences between vesicles treated with or without alpha factor. We are currently obtaining sufficient quantities of purified vesicle to analyze by LC/MS/MS.

## Endocytosis I (428-448)

428

### Real-time Fluorescence Analysis of Receptor-mediated Endocytosis

J. Y. Toshima, J. Toshima, M. Kaksonen, A. C. Martin, D. S. King, D. G. Drubin; Department of California Berkeley, University of California Berkeley, Berkeley, CA

In both yeast and mammals, endocytic vesicle internalization is a dynamic process that requires coordinated interactions between plasma membrane proteins and the actin cytoskeleton. Much progress defining the order and timing of endocytic internalization events has come as a result of real-time, live-cell fluorescence microscopy. While the availability of numerous endocytic mutants makes yeast an especially promising organism for functional analysis of endocytic dynamics, a serious limitation has been the lack of a fluorescent cargo for receptor-mediated endocytosis, whose transit through the internalization pathway can be tracked visually. In this study, we succeeded in synthesizing fluorescently labeled mating pheromone derivatives, and discovered previously unknown spatio-temporal features of receptor-mediated endocytosis. These labeled pheromones maintained biological activity as assessed by induction of mating morphology in a cells, although the activities were about 25-50-fold less than for for wild-type alpha-factor. Using this novel endocytic marker, we found that actin patches were sites of receptor-mediated endocytic internalization. In addition, we found that endocytic proteins first assembled into patches on the plasma membrane, then alpha-factor associated with the patches, and finally internalization occurred concomitant with actin assembly at patches. Furthermore, endocytic vesicles moved on actin cables to facilitate efficient trafficking between the plasma membrane and endosomes, while endosomes could also actively move toward endocytic sites on actin cables to capture vesicles as they were being released from the plasma membrane. These studies establish that multiple steps during receptor-mediated endocytosis are mediated by filamentous actin, and that an endocytic ligand gets recruited to the endocytic machinery, and not vice versa.

429

### System-wide Live-cell Imaging of Actin-mediated Endocytosis

M. Kaksonen, C. P. Toret, D. G. Drubin; Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Actin is emerging as a vital component of the endocytic machinery in organisms from fungi to mammals. Actin filaments are polymerized transiently at endocytic sites during vesicle budding, and this polymerization has been suggested to provide force for plasma membrane invagination and vesicle movement. We explored the mechanisms underlying this actin-mediated process by analyzing the dynamic behavior of GFP-fusion proteins marking either the endocytic coat or actin filaments in 60 mutant budding yeast strains each lacking one endocytic gene. This screen revealed phenotypes for 14 proteins. Dynamics of ten of these 14 proteins were examined by live-cell imaging. Quantitative live-cell analysis of both protein dynamics and corresponding deletion phenotypes suggested roles for these proteins at different steps during actin-mediated endocytic internalization. We found that clathrin, Sla1p and End3p play a role in initiating the assembly of the actin-based endocytic machinery, Bbc1p and capping protein (Cap1p and Cap2p) regulate the speed of the actin-driven coat movement, fimbrin (Sac6p) couples actin polymerization to coat movement, amphiphysins (Rvs161p and Rvs167p) play a role in vesicle scission and Abp1p in disassembly of the endocytic machinery. We also discovered that simultaneous deletion of both *BBC1* and *SLA1* genes caused formation of giant dynamic actin protrusions at endocytic sites. Remarkably, these protrusions were still able to drive endocytosis. Due to the large size of these actin structures (~10x normal) we were able to visualize membrane invagination and vesicle formation in relation to the actin protrusions in living cells. Furthermore, using local photobleaching of actin-GFP we showed that the actin protrusion that pushes the newly formed vesicle was polymerized at the plasma membrane, not at the vesicle surface. This mechanism is opposite to the classical actin-rocketing model for *Listeria* and endosome motility.

430

### Proteomic Analysis of Immunoisolated Apical Recycling Vesicles

L. A. Lapierre,[1,2] K. Avant,[1,2] J. S. Lowe,[3] A. L. Ham,[4] S. Hill,[4] J. R. Goldenring[1,2,3]; [1]Depart. of Surgery and Epithelial Biology Program, Vanderbilt Univ. Medical Center, Nashville, TN, [2]Nashville VA Medical Center, Nashville, TN, [3]Depart. of Cell and Developmental Biology, Vanderbilt Univ. Medical Center, Nashville, TN, [4]Depart. of Biochemistry and Mass Spectrometry Research Center, Vanderbilt Univ. Medical Center, Nashville, TN

The apical recycling system is responsible for the timely and accurate spatial trafficking of many receptor and transporters in polarized epithelial cells. To understand better the regulatory control of apical recycling in polarized cells we have used a proteomic approach to characterize immunoisolated tubulovesicular recycling membranes from the parietal cells of human stomach. Using a monoclonal antibody against the α-subunit of the gastric H/K-ATPase, we immunoisolated the vesicles out of an enriched tubulovesicle compartment from the resting human gastric mucosa of four organ donors. Proteins were eluted from the beads with 1% CHAPS and tryptic peptides were analyzed by LC-MS-MS. We identified and validated by western blots many of the components previously identified on recycling vesicles including the small GTPases Rab11 and Rab25 as well as Syntaxin 3, SCAMPs and VAMP2. We have now identified by proteomic analysis of immunoisolated vesicles Rabs 4 and 10 as well as VAMP8, syntaxins 7 and 12/13, pantophysin and the human homolog of YOP1p, DP1/TB2 as well as Annexins and S100-A10. Transfection of a stable GFP-Rab11a expressing MDCK cell line with Vamp8 or pantophysin caused a collapse of the GFP-Rab11a compartment, this effect was also observed, but less dramatic in syntaxin 7 and VAMP2 transfected cells. While overexpression of DsRed2-pantophysin altered the GFP-Rab11a compartment, it did not co-localize with the GFP-Rab11a but was localized to an adjacent tubular compartment. This proteomic examination of apical recycling vesicles from parietal cells has led to insights into membrane recycling regulators in epithelial cells.

431

**A Role of the Breakpoint Cluster Region Protein in Endocytosis**

E. K. Malmberg,[1,2] Y. Hou,[2] J. R. Riordan,[2] G. C. Hansson,[1] M. Gentzsch[2]; [1]Medical Biochemistry, Göteborg University, Gothenburg, Sweden, [2]Mayo Clinic College of Medicine, Scottsdale, AZ

The Breakpoint cluster region (Bcr) protein is multifunctional, participating in various signalling pathways. In addition to an N-terminal oligomerization domain it has four structural domains including a serine/threonine kinase, a tandem Dbl homology/Pleckstrin homology (DH/PH) domain characteristic of RhoGEFs, a GAP domain and a $Ca^{2+}$ -dependent phospholipid C2 domain in the C-terminus. Both GEF and GAP activities target some of the same Rho GTPase family members providing the possibility of bi-directional regulation of Rho controlled signalling pathways. These control many cellular functions involving the cytoskeleton and vesicular traffic. In addition to the previously known functional domains we found that Bcr also contains a clathrin binding box (CBB) as well as a consensus sequence for interaction with the AP-2 clathrin adapter complex. We show that Bcr binds both to clathrin and the α-adaptin subunit of AP-2. Although Bcr appears as an abundant cytoplasmic protein in most cells both the PH and C2 domains might be expected to mediate membrane association. This was confirmed with immunofluorescence microscopy on polarized human epithelial cells which showed a significant accumulation of Bcr at the plasma membrane. Isolation of clathrin coated vesicles from human cells of epithelial origin (HEK293) revealed that Bcr is enriched on these organelles. In BHK-21 cells stably overexpressing wild type Bcr, endocytosis was greatly exaggerated compared to wt BHK-21 cells, when measured by dextran Alexa-488 internalization analyzed by confocal microscopy. To conclude, we suggest that Bcr is acting as an endocytic adapter protein in clathrin mediated endocytosis.

432

**Regulation of "Constitutive" Endocytosis by Dynamin and Cortactin Phosphorylation**

H. Cao, J. Chen, A. E. Kruchten, M. A. McNiven; Center for Basic Research in Digestive Diseases and Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN

The mechanisms by which epithelial cells regulate clathrin-mediated endocytosis (CME) are poorly defined. In fact, the endocytic internalization of many ligands such as transferrin is considered a constitutive process that occurs continuously without regulatory constraints. In this study, we demonstrate for the first time that endocytosis of the transferrin receptor is a regulated process that activates c-Src and, subsequently, multiple components of the endocytic machinery including the large GTPase dynamin (Dyn2) and its associated actin-binding protein cortactin. Immunoprecipitation of c-Src from cultured NIH/3T3 cells showed substantial levels of co-precipitated cortactin and Dyn2. In support of this, purified, recombinant Dyn2 and cortactin were phosphorylated by c-Src in vitro while Dyn2 and cortactin proteins with tyrosine residues mutated to phenylalanine (Dyn2:Y231F and Y597F and Cort:Y384F, Y429F, and Y445F) were not phosphorylated. When expressed in a cultured hepatocyte cell line (Clone 9), these mutant proteins significantly attenuated CME of transferrin. Consistent with these findings, expression of either a kinase dead (K297M) or a constitutively inactive (Y419F) form of c-Src also inhibited transferrin internalization. Finally, Clone 9 cells exposed to transferrin showed a significant increase in Dyn2 and cortactin phosphorylation compared to untreated cells, an increase exceeding that observed by cells treated with EGF. These findings are the first to implicate a c-Src-mediated endocytic cascade in what was previously presumed to be a non-regulated receptor-ligand internalization process.

433

**Functional Characterization of the Interaction between the Endocytic Sorting Adaptor Stonin 2 and Synaptotagmin**

N. Jung, M. K. Diril, V. Haucke; Institut fuer Chemie - Biochemie, Freie Universitaet, Berlin, Germany

Stonins 1 and 2 are the only mammalian orthologues of the *Drosophila melanogaster* stoned B protein, an endocytic protein implicated in synaptic vesicle recycling. Stonins interact with a variety of proteins involved in clathrin-mediated endocytosis (CME) including the adaptor AP2, Eps15, and intersectin. We have mapped the AP2 binding region to tandemly repeated WxxF motifs within the amino-terminal domain of stonin 2. Its carboxy-terminal μ-homology domain interacts with the C2B domain of several synaptotagmin family members. Pull down and co-immunoprecipitation experiments indicate a role for a basic cluster of amino acids within the C2B domain of synaptotagmin I in stonin 2 binding. Surprisingly we found that in addition to C2B also the C2A domain of synaptotagmin 1 is capable of interacting with stonin 2. The ability of stonin 2 to bind to AP2 and synaptotagmin both *in vitro* and *in vivo* suggests a function for stonin 2 in clathrin/AP2-mediated synaptotagmin internalization. In fact, we found that transfected synaptotagmin 1 located to the plasma membrane in fibroblasts is endocytosed in the presence of stonin 2. Current efforts are directed at dissecting the exact sorting mechanism involved in this pathway and at testing its relevance for vesicle cycling in the brain.

434

**The N and C Termini of Clathrin Light Chain Interact in a Calcium-calmodulin-dependent Manner: Implications for Neuronal Clathrin Trafficking**

S. L. Newmyer, V. Manalo, F. M. Brodsky; Hooper Foundation, University of California, San Francisco, San Francisco, CA

Clathrin-coated vesicle trafficking mediates diverse cellular functions, ranging from down-regulation of cell surface receptors to recycling synaptic vesicles. Different adaptor proteins have been demonstrated to promote various clathrin trafficking activities. However, clathrin is expressed in a tissue-dependent manner and, therefore, may also contribute to membrane trafficking diversity. Vertebrates express two light chains, LCa and brain-enriched LCb. Additionally, neuronal LCs contain 18-30 amino acid inserts through alternative splicing. In vitro, LCs negatively regulate coat assembly through an invariant LC N terminal EED motif. These residues may suppress heavy chain (HC) assembly by either binding HC directly or interacting with another LC region to modulate HC indirectly. Evidence exists that LC-LC interactions may occur. The present study aims to establish whether and how LC-LC interactions occur to understand how changing LC expression facilitates tissue-specific clathrin trafficking. Through co-precipitation assays utilizing GST-fused LCs and LC fragments, we find that LCs, indeed, bind one another. Our analysis identifies C terminal LCa residues 189-248, which includes a region of previously unknown function and the C terminal calmodulin binding domain, as the minimal fragment that binds the N-terminus. Addition of the neuronal insert, residues 158-188, within the GST-LC fragment enhances neuronal and non-neuronal LC co-precipitation. Thus, the neuronal insert may modulate LC-LC interactions. Calcium-calmodulin

inhibits LC-LC binding. Placing our findings in the context of biochemical HC assembly assays, we conclude that N-C terminal LC-LC interactions suppress coat assembly. Moreover the ability of calmodulin to disrupt LC-LC interactions suggests a potential calcium-regulated mechanism to allow rapid compensatory endocytosis at the firing synapse. Currently we are expressing LC fragments and mutants within hippocampal neurons to disrupt LC-LC binding and elucidate how this interaction modulates receptor-mediated versus compensatory endocytosis

435

**Interactions between the Yeast Endocytic Scaffold Pan1 and the Type I Myosins Myo3/5 Contribute to a Late Stage of Endocytosis**

S. L. Barker, B. Wendland; Department of Biology, The Johns Hopkins University, Baltimore, MD

The endocytic scaffold Pan1 of *Saccharomyces cerevisiae* is comprised of three regions; the amino-terminal adaptor-binding region, the central oligomerization region, and the carboxy-terminal F-actin-binding and Arp2/3 stimulation region. The carboxy-terminal region also contains an uncharacterized proline-rich domain (PRD) which contains ligand motifs predicted to bind to SH3 domains. We conducted a yeast two-hybrid screen to identify binding partners for the PRD and isolated clones corresponding to the SH3 domains of the type I myosins, Myo3 and Myo5. In vitro binding and in vivo co-immunoprecipitation assays validated this interaction. Two-color real-time fluorescence microscopy revealed the relative timing of recruitment of Pan1-GFP and Myo3/5-RFP to nascent endocytic sites; we found that Myo3/5 join Pan1 at cortical patches at a very late stage of internalization, just prior to the brief inward movement of Pan1 and its disassembly. Consistent with an in vivo role for the carboxy-terminal interactions of Pan1 and Myo3/5, we found that the widely used *pan1-20* temperature sensitive allele corresponds to a disruption of the PRD due to a frame-shift mutation in the open reading frame of the domain. *pan1-20* cells have an increased number of invaginations at the plasma membrane, consistent with a defect in vesicle scission. The pan1-20 protein has reduced affinity for Myo3/5, and *pan1-20* cells are deficient in recruiting Myo3/5 to endocytic sites. We propose that the interaction of Pan1 and the type I myosins promotes a late stage of endocytic internalization to facilitate endocytic vesicle scission.

436

**Recruitment of EH Domain-containing Endocytic Scaffold Proteins by Clathrin Adaptors is a Required Step in Endocytosis**

L. Maldonado-Baez, B. Wendland; Department of Biology, The Johns Hopkins University, Baltimore, MD

Endocytic adaptor proteins are involved in cargo selection and recruitment of other members of the machinery. Our previous work has demonstrated that the yeast epsins, Ent1 and Ent2, which are putative adaptors, are essential for viability in yeast. We have mapped the essential function of the epsins to the epsin N-terminal homology (ENTH) domain; the endocytic function resides in the C-terminus, which contains the characterized endocytic protein interaction motifs. The yeast Yap1801 and Yap1802 proteins, homologs of mammalian AP180/CALM, are structurally similar to the epsins. To test possible redundancy between these proteins as cargo adaptors in endocytosis, we created a quadruple delete mutant lacking the four adaptors and carrying a plasmid encoding the ENT1 ENTH domain (ENTH1) as the only source of epsin. We found that the ENTH1 domain complements quadruple delete cells, as we previously observed in ent1Δent2Δ cells. However, unlike ent1Δent2Δ+ENTH1, quadruple mutants+ENTH1 are inviable at 37°C and defective for endocytosis of the transmembrane receptor Ste3 at 30°C. Adding back either of the Yap180s restores growth at 37°C, suggesting that the Yap180s can replace a function of the epsin C-terminus. Ent1 truncations identified the minimal region of epsin required for endocytosis and 37°C growth in the absence of the Yap180; it must include the NPF tripeptide-containing motifs that bind to EH domain-containing endocytic scaffold proteins. Quadruple mutants+ENTH1 were rescued when co-transformed with an NPF-containing truncation of Yap1802, but not with a fragment lacking the NPF motifs. The evidence indicates an important role for interactions between adaptors and scaffolds for endocytosis and viability at 37°C. These results suggest that functional redundancy resides in adaptor C-termini, and that the NPF-motif/EH domain interaction is required for adaptors to recruit scaffolds to allow completion of endocytosis.

437

**BAR Domains of Human DIP13α/APPL and DIP13β Mediate Homotypic and Heterotypic Protein-Protein Interactions**

H. J. Chial, R. Wu, Y. Q. Chen; Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, NC

Human DIP13α/APPL/APPL1 and DIP13β/APPL2 are members of a distinct family of proteins that contain a predicted N-terminal BAR (Bin/Amphiphysin/Rvs) domain, a central pleckstrin homology (PH) domain, and a C-terminal phosphotyrosine binding (PTB) domain. DIP13α exhibits PTB domain-mediated interaction with the intracellular domain of the deleted in colorectal cancer (DCC) protein and is required for DCC-mediated apoptosis (Liu et al., 2002). DIP13α also interacts with AKT protein kinases (Mitsuuchi et al., 1999), phosphatidylinositol 3-kinase (PI3K) subunits (Mitsuuchi et al., 1999), and the human follicle-stimulating hormone receptor (Nechamen et al., 2004). DIP13α and DIP13β are also RAB5 effectors that interact with GTP-bound RAB5 on a subset of endosomes (Miaczynska et al., 2004). Following epidermal growth factor (EGF) stimulation, DIP13α undergoes RAB5-dependent translocation from endosomal membranes to the nucleus (Miaczynska et al., 2004). Based on these findings, DIP13 proteins are predicted to function in a novel endosome-mediated signaling pathway linking the cell surface to the nucleus. Interaction between DIP13 proteins and RAB5 requires the DIP13 BAR domain and adjacent PH domain (Miaczynska et al., 2004). BAR domains are known for their ability to form crescent-shaped dimers capable of sensing and inducing membrane curvature. Using co-immunoprecipitation experiments, we have detected DIP13α-DIP13α, DIP13β-DIP13β, and DIP13α-DIP13β protein-protein interactions *in vivo*. We used the yeast two-hybrid system and deletion analyses to demonstrate that the DIP13α and DIP13β BAR domains are necessary and sufficient for mediating all homotypic and heterotypic DIP13-DIP13 protein-protein interactions. Taken together, our results show that DIP13α and DIP13β BAR domains function to promote homotypic and heterotypic DIP13-DIP13 interactions, and suggest that these interactions may play a role in DCC- and endosome-mediated signaling.

438

**The Signaling Adaptor Grb2 is Required for cMet Internalization**

N. Li,[1] K. Ireton,[2] L. A. Elferink[1]; [1]Dept. of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX, [2]Biomolecular Science Center, Department of Molecular Biology and Microbiology, University of Central Florida, Orlando, FL

Increased cMet signaling underlies several human cancers, correlating closely with metastatic tendency, angiogenesis and poor prognosis. In normal cells, cMet activation is tightly regulated by signaling cascades and endocytosis. However, it remains unclear how cMet activation is

mechanistically linked to receptor endocytosis. Activation of the cMet receptor by ligand binding leads to recruitment of multiple signaling proteins, including the adaptors Grb2 and Gab1, to two 'docking site' tyrosines (Y1349 and Y1356), located in the C-terminal tail of the receptor. Since these tandem tyrosines are essential for the oncogenic activity of activated cMet, we examined their role in receptor endocytosis. In contrast to the situation with wild-type cMet, a mutant receptor in which both tyrosines 1349 and 1356 were changed to phenylalanine (Y1349,1356F) failed to undergo ligand-dependent internalization. In Y1349, 1356F expressing cells, cMet ubiquitination and phosphorylation of Gab1 and the E3 ubiquitin ligase Cbl were decreased, with negligible change in receptor tyrosine phosphorylation. Multiple lines of evidence suggest that the defect in Cbl phosphorylation and endocytosis of the mutant receptor were due to effects on Grb2, and not Gab1, recruitment. First, depletion of Grb2 by siRNA or inhibition of Grb2 recruitment through the use of dominant negative mutants impaired receptor ubiquitination and internalization. In contrast, genetic inactivation or siRNA-mediated knock-down of Gab1 did not block cMet endocytosis. Taken together, these results indicate that internalization of cMet requires Grb2, but not the Gab1 adaptor. Since Grb2 is constitutively associated with Cbl, it seems likely that the role of this adaptor in endocytosis is mediated in part, by Grb2-Cbl complexes. Future studies on the contributions of Grb2 and Cbl in the attenuation of cMet signaling through receptor internalization and degradation, will likely contribute to better understanding of mechanisms of oncogenesis.

439

**Post-translational Modification of the P Selectin Endocytic Motif Controls Receptor Degradation**

M. S. Roberts, D. Cutler; Laboratory for Molecular Cell Biology, University College London, London, United Kingdom

P selectin is a type 1 transmembrane protein found in the secretory granules of endothelial cells (Weibel Palade bodies). Following stimulation with proinflammtory mediators, P selectin is redistributed to the plasma membrane where it can interact with a counter-receptor (PSGL1) on leukocytes that leads to leukocyte rolling. Leukocytes stop rolling following chemo-attractant-mediated integrin activation, and then cross the endothelial barrier into the tissue where they carry out their inflammatory function. Unregulated long-term residence at the plasma membrane would lead to persistent leukocyte recruitment and chronic inflammation, but its rapid internalisation and subsequent endocytic trafficking is such as to preclude its uncontrolled return to the plasma membrane. We have identified an endocytic motif contained within the cytoplasmic tail of P selectin that is essential to traffic between early and late endosomes. This is a major sorting step determining the fate of P selectin, as P selectin may subsequently be degraded in the lysosome or recycled back to Weibel Palade bodies ready to act in a second round of inflammation. We have been studying the molecular mechanisms controlling this process and we determined that the post-translational modification of the endocytic motif of P selectin is essential to this sorting event.

440

**New Insights into Insulin-mediated Modulation of GLUT4 Endocytosis in Adipocytes**

V. Blot, T. McGraw; Biochemistry, Weill Medical College of Cornell, New York, NY

Insulin induces a large redistribution of the GLUT4 glucose transporter from intracellular storage compartments to the plasma membrane by increasing the rate of GLUT4 exocytosis and decreasing its rate of endocytosis. The effects of insulin on GLUT4 exocytosis are well described; the effects on endocytosis, however, are still controversial. Using quantitative fluorescence microscopy, we measured the internalization rates of HA-GLUT4-GFP reporter molecules in adipocytes in both basal and insulin-stimulated adipocytes. Insulin stimulation reduced GLUT4 internalization rate by 2 to 3-fold, whereas it increased the internalization of the transferrin receptor by about 1.5 fold. We analyzed the effect of mutations of the $F^{5}$QQI, $L^{489}$L and $E^{499}$LEY GLUT4 trafficking motifs on basal and insulin-modulated endocytosis. We discovered the following: 1) the $F^{5}$QQI motif is necessary for efficient GLUT4 internalization in the presence of insulin stimulation but dispensable in the basal state, 2) the $L^{489}$L motif is dispensable for internalization in both the basal and the insulin-stimulated states and 3) mutation of the $E^{499}$LEY motif did not affect internalization in basal adipocytes. This mutation, however, prevents the decrease in GLUT4 internalization upon insulin stimulation, indicating that insulin-inhibition of GLUT4 endocytosis requires the $E^{499}$LEY motif. Finally, only the combined mutation of both the $F^{5}$QQI and the $E^{499}$LEY was found to reduce GLUT4 internalization in basal adipocytes. This study starts to unravel, at a molecular level, the effect of insulin on GLUT4 internalization. Our results support a model in which insulin specifically modulates GLUT4 internalization rate by regulating the function of specific GLUT4 endocytosis motifs.

441

**Analysis of the Endocytic Behavior and Intracellular Localization of the C-type Lectin DC-SIGN**

H. Nassanian, B. Lee; Microbiology, Immunology & Molecular Genetics, UCLA, Los Angeles, CA

Dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) is a C-type lectin receptor expressed by DCs implicated in the binding and transfer of human immunodeficiency virus (HIV). As a type II integral membrane protein, DC-SIGN has an extracellular C-terminal carbohydrate recognition domain, a hydrophobic transmembrane domain, and an N-terminal cytoplasmic domain (CD). The CD has three putative internalization/sorting motifs that may play a role in receptor endocytosis and trafficking, including a tyrosine-based motif (YKSL), a dileucine motif (LL), and an acidic cluster (EEE). By utilizing a cell surface biotinylation and endocytosis assay followed by an ELISA, the contribution of each motif in DC-SIGN endocytosis was examined in 293T transfectants. We have found that the dileucine motif accounts for most of the active endocytosis as both LL and YKSL/LL double mutants exhibited the same endocytic defect when compared to wild-type DC-SIGN. Endocytosis reached a plateau at 20 minutes, where 55% of wild-type DC-SIGN was endocytosed. Similar experiments were used to study the rate of DC-SIGN endocytosis in primary monocyte-derived dendritic cells (MDDCs). Indeed, endocytosis was more rapid, reaching a plateau in 10 minutes. Due to our ELISA-based format, the endocytic rate of DC-SIGN can be compared to other cell surface markers, like MHC class II and other DC lectins. The intracellular localization of DC-SIGN and its mutants was also studied by antibody uptake experiments analyzed by immunofluorescence staining. Microscopy data shows similar patterns with the dileucine and dileucine/tyrosine mutants being defective in internalization as compared to wild-type DC-SIGN. Co-localization of DC-SIGN and its mutants with organelle-specific markers will further explain the intracellular trafficking of this receptor. We will determine if the LL motif is active in MDDCs by competitive inhibition (via peptide transfection) with mutant peptides targeting these motifs in DC-SIGN.

442

**Sulfatide-facilitated Internalization of Cobra Cardiotoxin**

C. Wang, S. Lee, W. Wu; Department of Life Sciences, Hsinchu, Taiwan Republic of China

Amphiphilic β-sheet cobra cardiotoxins have recently been shown to target mitochondria and disrupt its network to regulate CTX-induced cell death pathway, but the mechanism involved in its internalization remains illusive. Herein, we show that the internalization of CTX A3, the major cobra cardiotoxin from Taiwan cobra, is a temperature-dependent, sulfatide-facilitated, and cholestyerol-inhibitory membrane de-limited process in H9C2 myoblast. In contrast to the internalization of other glycosphingolipid or glycosphingolipid binding toxins by a clathrin-independent, dynamin-dependent and cholesterol-sensitive caveolae/raft-dependent endycytosis, CTX A3 exploits a caveolin-, clathrin-, dynamin- and ATP-independent pathway. The process, however, can be promoted by heparan sulfate, with either cell surface bound or free form, when its carbohydrate chain length becomes longer than 10 residues. Based on the properties of CTX A3 action on model membranes and glycosaminoglycans, we suggest that heparan sulfate-promoted dimerization of CTX A3 with membrane favorable conformation may facilitate the binding of CTX A3 to sulfatide lipid phase and induce ATP-independent endocytosis. CTX A3 binding to sulfatide domain can be inhibited by enhancing cholesterol concentration. It also triggers a pathway shift of exogenous sulfatide internalization from a high cholesterol to a low cholesterol-mediated process for CTX A3 to be delivered to mitochondria and regulate CTX cytotoxicity.

443

**Clathrin Dependent Endocytic Trafficking of the ClC-3 Chloride Channel**

Z. Zhao, S. A. Weinman; Neuroscience and Cell Biology, Univ. of Texas Medical Branch at Galveston, Galveston, TX

ClC-3 is an intracellular chloride channel required for acidification of synaptic vesicles and endosomes. We have previously shown that a small portion of ClC-3 is transiently present on the plasma membrane and undergoes rapid endocytosis. The **OBJECTIVE** of this study was to determine whether plasma membrane ClC-3 represents a trafficking intermediate for intracellular localization the mechanism of its internalization. **METHODS:** A ClC-3 derivative was constructed by adding an HA epitope at position 1217 of an external loop. COS-7 cells were transfected with Fugene6 and observed under epi and confocal microscopy. N terminal and deletion fusion proteins were used in GST pull down assays. **RESULTS:** Incubation of live cells with anti-HA antibody specifically labeled plasma membrane localized ClC-3 where it colocalized with clathrin. After 15-30 min chase at 37°C, ClC-3 was primarily localized in small endocytic vesicles as evidenced by colocalization with clathrin and transferrin receptor. After 120min chase, ClC-3 was localized exclusively in large centrally located membrane structures that localized with lysosomal markers but not with clathrin. In GST pull down assays the N terminal of ClC-3 bound to clathrin heavy chain and AP-2 but not caveolin. Deletion of a segment within the cytosolic N terminal (amino acid 12-30) decreased endocytosis and increased steady state persistence at the plasma membrane. This deletion also abolished interaction with clathrin but not AP-2. **CONCLUSION:** ClC-3 targeting to endosomes and lysosomes occurs primarily via the indirect trafficking route where plasma membrane insertion and clathrin-dependent endocytosis are critical. An N terminal segment is responsible for clathrin binding.

444

**Differential Phosphorylation and Dephosphorylation Kinetics of β2-adrenergic Receptor Sites Ser 262 and Ser 355,356**

V. Iyer,[1] T. M. Tran,[2] E. Foster,[1] R. B. Clark,[2] B. J. Knoll[1]; [1]Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, TX, [2]Integrative Biology and Pharmacology, University of Texas Medical School, Houston, TX

G protein-coupled $\beta_2$-adrenergic receptors ($\beta_2$ARs) are rapidly desensitized by phosphorylation of Ser262 by protein kinase A (PKA) and of Ser355,356 by G protein-coupled receptor kinase (GRK). We sought to determine whether the phosphorylation and subsequent dephosphorylation of these sites had similar kinetics and requirements for receptor endocytosis. The phosphorylation of the PKA and GRK sites were measured using antibodies that recognize pSer262 and pSer355,356. Endocytosis in stably transfected HEK293 cells was blocked by inducible expression of dominant negative dynamin-1 K44A or by treatment with hypertonic sucrose. The extent and kinetics of phosphorylation of the GRK site Ser355,356 during treatment with the strong agonist isoproterenol (ISO; 10μM) were unaffected by dynamin-1 K44A expression, and minimally by hypertonic sucrose. Under control conditions, the phosphorylation of the PKA site Ser262 during 10 μM ISO treatment peaked after 2 mins and then rapidly declined, but inhibition of endocytosis enhanced and prolonged this phosphorylation. Treatment with 300 pM ISO, where there is no receptor endocytosis or phosphorylation by GRK, also caused prolonged PKA site phosphorylation. The dephosphorylation of these sites was measured after removal of agonist and the addition of antagonist. Significant dephosphorylation of both phosphoserines was observed under conditions of very low endocytosis. The results indicate that the kinetics of $\beta_2$AR phosphorylation by GRK and PKA are distinct and differentially affected by endocytosis, and that receptor dephosphorylation can occur either at the plasma membrane or in internal compartments. Supported by grants 0455072Y from the American Heart Association, Texas Affiliate (BJK) and GM031208 from the NIH (RBC).

445

**Proteolytic Processing of the 315 kDa Human HARE/Stab2 Hyaluronan Receptor**

E. N. Harris, P. H. Weigel; Dept. of Biochemistry, University of Oklahoma HSC, Oklahoma City, OK

The human Hyaluronic Acid Receptor for Endocytosis (HARE; also called Stabilin-2) is the predominant endocytic clearance receptor for circulating hyaluronan (HA) and other glycosaminoglycans that originate from ECMs of tissues throughout the body. HARE is highly expressed in the sinusoidal endothelial cells of lymph node, spleen, and liver. The full-length human *hare* cDNA (7653 bp) encodes a glycoprotein of 2551 amino acids that migrates in SDS-PAGE to approximately 315 kDa. Immunopurification from human or rat spleen and liver revealed that HARE exists as two receptor isoforms (~315 kDa and ~190 kDa) that are likely the result of specific proteolytic cleavage of the full-length protein. The N-terminus of the 190 kDa spleen isoform corresponds to proteolytic cleavage before serine 1136 in full-length hHARE and does not contain consensus sequences for any known proteases. The same pattern of proteolytic processing is also observed in 293 cell lines, which stably express the full-length *hare* cDNA. Pulse-chase experiments using $^{35}$S-Cys/Met reveal that full-length 315 kDa HARE receptor is synthesized, glycosylated in the Golgi, and then presented on the cell surface before the appearance of the 190 kDa HARE. N-terminally labeled GFP-190 HARE is also cleaved to produce free GFP and the 190 HARE. Conversely, full-length HARE lacking the cytoplasmic and transmembrane domains (a secreted ectodomain) is not proteolytically cleaved, indicating that membrane anchorage is vital for receptor processing. We conclude that the proteolytic

cleavage of full-length HARE to create the second smaller isoform is a natural non-artifactual process and that both isoforms are actively engaged in glycosaminoglycan clearance. (Supported by NIH GM69961 and F32 GM070262-01A1).

446

**Glycosaminoglycan Binding and Endocytosis Mediated by Cells Expressing Recombinant Human HARE/Stab2 Receptor**

E. N. Harris, J. A. Weigel, S. Kiosseva, P. H. Weigel; Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

The Hyaluronic Acid (HA) Receptor for Endocytosis (HARE; also designated Stabilin-2) mediates systemic clearance of glycosaminoglycans from the circulatory and lymphatic systems. Two membrane-bound HARE iso-receptors (a full-length ~315 kDa form and a truncated ~190 kDa form derived by proteolysis) are highly expressed in sinusoidal endothelial cells of liver, lymph node, and spleen. Multiple clones of Flp-In 293 cell lines stably expressing full-length hHARE cDNA make both receptor isoforms (315 and 190) in culture, although the clones differ in total receptor expression levels and in their HA binding, endocytosis and degradation activities. Both receptor isoforms cross-react with three anti-rat HARE monoclonal antibodies, which recognize their extracellular domains and partially block specific endocytosis of HA by HARE. We also developed cell lines that secrete full-length HARE without the transmembrane and cytoplasmic domains. The secreted 315 kDa HARE ecto-domain was purified from media via metal-chelate chromatography. In an ELISA format, the ecto-domain binds to HA with a higher affinity than to chondroitin sulfates A-E. Unlabeled chondroitin sulfates A-E also blocked, to different degrees, HA endocytosis by 315 kDa HARE stable cell lines. To determine the cellular distribution of the two receptor isoforms, we are currently biotinylating intact or permeabilized cells and then examining the labeled 315 kDa and 190 kDa HARE proteins by Western analysis using streptavidin-HRP. Preliminary results suggest that more receptors reside within intracellular compartments than on the cell surface. We conclude that both the 315 kDa and 190 kDa HARE receptor isoforms can mediate glycosaminoglycan clearance via endocytosis and contribute to normal homeostasis. (Supported by NIH GM69961 and F32 GM070262).

447

**Enterocyte Toll Like Receptor-4 Mediates Phagocytosis and Translocation across the Intestinal Barrier**

C. L. Leaphart,[1] M. D. Neal,[1] J. Prince,[1] R. Levi,[1] J. Li,[1] T. Billiar,[1] S. Cetin,[1] S. Watkins,[2] D. J. Hackam[1]; [1]Surgery, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Cell Biology, University of Pittsburgh, Pittsburgh, PA

Translocation of bacteria across the intestinal barrier is important in the pathogenesis of systemic sepsis, although the mechanisms by which bacterial translocation occurs remain largely unknown. We hypothesized that bacterial translocation across the intact barrier occurs after internalization of the bacteria by enterocytes in a process resembling phagocytosis, and that toll like receptor 4 (TLR4) is required for this process. We now show that FcRγIIa-transfected enterocytes can internalize IgG-opsonized erythrocytes into actin rich cups, confirming they have the molecular machinery required for phagocytosis. We further show that enterocytes can internalize *E. coli* into phagosomes, that the bacteria remain viable intracellularly, and that TLR4 is required for this process. TLR4 signaling was found to be necessary and sufficient for phagocytosis by epithelial cells as rat IEC-6 cells were able to internalize LPS-coated but not uncoated latex particles, and TLR4-transfected HEK-293 cells acquired the capacity to internalize *E. coli* whereas non-transfected HEK-293 did not. Strikingly, the internalization of gram negative bacteria into enterocytes in vivo and translocation of bacteria across the intestinal epithelium to mesenteric lymph nodes were significantly greater in wild-type mice as compared to mice with mutations in TLR4. These data suggest a novel mechanism by which bacterial translocation occurs, and suggest a critical role for TLR4 in the phagocytosis of bacteria by enterocytes in this process.

448

**Failure of Endocytosis in Experimentally Produced Amicronucleate Tetrahymena Cells**

R. Tsuchiya, J. Wolfe; Biology, Wesleyan University, Middletown, CT

It is now possible to experimentally induce the resorption of nuclei en masse in living cells. This was accomplished with *Tetrahymena*, a ciliated protozoan which has a large macronucleus controlling the metabolism of the cell and a small micronucleus that shows no signs of gene exppression. After pairing of mating types the micronucleus undergoes an extraordinary elongation. When 2.5 hr conjugants were exposed to colchicine elongation was reversed, and the micronuclei collapsed to a spherical shape. But then, collapsed micronuclei started to disappear. Astonishingly, after 5 hours of exposure to colchicine, about 50% of the paired cells lost their micronuclei. Elimination occurred by a lysosome-mediated mechanism suggestive of autophagy. Testing for growth of amicronucleates, we found that it was not possible, or was very rare. Suspecting that growth might be limited due to an inability to feed, we assayed for food vacuole formation, and found that cells identified as amicronucleate did not form food vacuoles. This observation raises the question of why micronuclear elimination results in a failure of phagocytosis. We are now exploring the possibility that micronuclear loss is accompanied by damage to the structure of the oral apparatus (OA) or its deep fiber. This would be consistent with observation by Haremaki et al (1996) who showed that amongst amicronucleates that were selected from cells that had divided under conditions of exposure to nocodazole, most showed some sort of damage to the OA. Relatedly, we are attempting to grow amicronucleate cells in an enriched growth medium known to support growth of mutant cells incapable of food vacuole formation. Finally, we should be able to harvest large numbers of amicronucleate cells with a magnetic separation of feeding cells that can phagocytose iron dextran particles, and probe why they do not grow.

## Protein Targeting (449-475)

449

**Mapping the Interaction of Mammalian SRP with Ribosome-Bound Nascent Chains**

A. L. Karamyshev,[1] I. Nilsson,[2] T. Hessa,[2] G. von Heijne,[2] A. E. Johnson[1]; [1]Medical Biochemistry & Genetics, Texas A&M University System Health Sciences Center, College Station, TX, [2]Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden

The signal recognition particle (SRP) recognizes signal sequences in nascent chains co-translationally and targets a ribosome•nascent chain complex (RNC) to the protein conducting channel (translocon) in the membrane of the endoplasmic reticulum (ER). Since the key structural elements of SRP recognition of signal sequence have not been defined at high resolution, we have examined the dependence of SRP binding to the

signal sequence as a function of its sequence. To assess the extent of SRP•signal sequence interaction, a photoreactive probe was incorporated into the middle of the preprolactin signal sequence by translating its mRNA (with an amber stop codon at the desired probe location) *in vitro* in the presence of $N^{\varepsilon}$-(5-azido-2 nitrobenzoyl)-Lys-$tRNA^{amb}$ (εANB-Lys-$tRNA^{amb}$) amber suppressor. A homogeneous population of RNC•SRPs containing a specific length of nascent chain was created by programming the translation with truncated mRNAs in the presence of purified canine SRP. Quantitative analysis of RNC•SRP photoadducts revealed that a decrease or increase in the charge at the N- or C-terminus of the signal sequence has only a mild effect on the association of SRP with nascent chains. However, alterations in the hydrophobic portion of the signal sequence severely affect SRP binding. Deletions of 1, 2, 3 or 4 hydrophobic leucines led to a gradual and finally complete inhibition of RNC•SRP complex formation. The photocrosslinking data correlate well with targeting efficiency and the extent of transport across the ER membrane. Thus, the hydrophobic core of the signal sequence is primarily responsible for its recognition and binding by SRP, while positive charges simply modulate the SRP•signal sequence affinity and its targeting to the translocon and translocation across the membrane.

450

**Folding of Nascent SecM within the Ribosome Exit Tunnel is Required for Translation Arrest**

C. A. Woolhead,[1] A. E. Johnson,[2] H. D. Bernstein[1]; [1]Genetics and Biochemistry Branch, National Institutes of Health, Bethesda, MD, [2]College of Medicine, Texas A&M University, College Station, TX

Recent work has shown that the *E. coli* "secretion monitor" protein (SecM) undergoes an elongation arrest which serves to regulate the synthesis of the downstream SecA protein. The arrest is signaled by an interaction between the C-terminus of nascent SecM and ribosomal proteins and rRNA that line the polypeptide exit tunnel. The arrest is relieved upon interaction of the SecM signal peptide with the translocase complex in the inner membrane. In this study we used fluorescent resonance energy transfer (FRET) to study the folding of nascent SecM inside the ribosomal tunnel. We found that the C-terminus of SecM has an elongated conformation, but adopts a more compact conformation as soon as the entire sequence that is required for elongation arrest has been synthesized. Mutations near the C-terminus of SecM, shown to abolish elongation arrest, did not inhibit the conformational change. In contrast, no conformational change was observed when wild-type SecM was synthesized on mutant ribosomes that fail to promote elongation arrest. In addition several interesting and novel mutations have been identified, in the C-terminus of SecM, which prevent compaction of the nascent chain but also preclude translation arrest. Taken together, these results suggest that folding of nascent SecM within the ribosome is not an intrinsic property of the polypeptide sequence but rather is induced by the ribosome at the point of translation arrest and that this compaction is necessary but not sufficient for elongation arrest to occur. We are currently investigating the interactions between the ribosomal proteins that line the tunnel walls and the nascent SecM polypeptide during the arrest process.

451

**Molecular Mechanism of a Small Molecule Inhibitor of Protein Translocation into the Endoplasmic Reticulum**

J. L. Garrison,[1] R. S. Hegde,[2] J. Taunton[1]; [1]Program in Chemistry and Chemical Biology and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA, [2]Cell Biology and Metabolism Branch, National Institutes of Health, Bethesda, MD

Secretory and membrane proteins constitute roughly one-quarter of total cellular protein, and represent a majority of current drug targets. In mammalian cells, biogenesis of secretory and membrane proteins requires threading of the nascent polypeptide through a channel in the endoplasmic reticulum (ER) membrane, a process known as cotranslational translocation. Targeting proteins to the translocation channel is mediated by remarkably diverse $NH_2$-terminal signal sequences that share little or no sequence homology and whose functional significance is not known. We have recently described the first specific small molecule inhibitor of cotranslational translocation, *cotransin*, and identified its target as the Sec61 protein complex, which forms the core of the channel. Remarkably, the sensitivity of proteins to cotransin is determined by their signal sequence. Cotransin inhibits translocation in a substrate-specific and signal sequence-discriminatory manner by interfering with the stable insertion of the nascent chain into the Sec61 channel. To further understand the molecular basis for cotransin's selectivity, we are using labeled analogs to directly measure the binding interaction between cotransin and the Sec61 complex. The molecular details of the cotransin-Sec61 interaction are likely to shed light on the mechanism by which unrelated signal sequences gate a communal translocation channel.

452

**Human Sec16 Homologs Influence tER Organization**

D. Bhattacharyya, B. S. Glick; Molecular Genetics & Cell Biology, University of Chicago, Chicago, IL

*Saccharomyces cerevisiae SEC16* encodes a 240-kD peripheral membrane protein that is involved in COPII vesicle budding from the ER. COPII vesicle assembly occurs at ER subdomains called transitional ER (tER) sites, but the mechanism that generates tER sites is unknown. Using another budding yeast, *Pichia pastoris*, we have obtained evidence that Sec16 functions as a determinant of tER organization. A *P. pastoris* cell contains only 2-6 tER sites, whereas a typical vertebrate cell contains several hundred tER sites. If the tER organizing function of Sec16 is conserved, vertebrate cells should contain a Sec16 homolog. Using BLAST searches with a central conserved region of yeast Sec16, we found two putative homologs in mammalian cells. hSec16S, the smaller of these proteins, is a 1061-residue protein that lacks a conserved C-terminal domain found in Sec16 homologs from other species. The larger Sec16 homolog, hSec16L, codes for a 2154-residue protein and has the conserved C-terminal domain. hSec16S and hSec16L are distinct genes but share 49% identity. In HeLa cells, GFP fusions to both hSec16S and hSec16L colocalized precisely with the COPII component Sec23 in a typical tER pattern. We found that siRNA-mediated knockdown of hSec16S or hSec16L severely disrupted tER sites, as judged by immunofluorescence with an anti-Sec23 antibody. These results suggest that both hSec16S and hSec16L are required for tER organization in mammalian cells. We are characterizing the interactions of these two proteins with each other and with other tER components. This study will help to elucidate the mechanism of tER site biogenesis in mammalian cells.

453

**Role of Transmembrane Proteins p25 and p23 in Endoplasmic Reticulum Localization of Protein Tyrosine Phosphatase TC48**

V. Gupta, G. Swarup; Centre for Cellular and Molecular Biology, Hyderabad, India

T-cell protein tyrosine phosphatase (TCPTP) gives rise to two splice forms: TC48, which is the ER membrane resident form, and TC45, a nuclear protein. The present study was undertaken to identify proteins that are involved in targeting TC48 to ER. We identified two TC48- interacting

proteins, p25 and p23 from a yeast two hybrid screen. p23 and p25 are members of a family of putative cargo receptors that are important for vesicular trafficking between Golgi-complex and ER. Both p23 as well as p25 associated with TC48 in Cos-1 cells co-transfected with p23 or p25 and TC48 as determined by coimmunoprecipitation. A significant amount of TC48 colocalized with ERGIC and Golgi-complex markers in addition to ER and nuclear membrane localization. Analysis of TC48 localization in time-course experiment as well as the use of pharmacological reagents demonstrated cycling of TC48 between Golgi-complex and ER. Co-expression with p25 enhanced ER-localization of TC48 while coexpression with p23 resulted in its trapping in irregular vesicular structures. C-terminal 40 amino acids of TC48 (376-415) were sufficient for interaction with p23 (but not with p25) and targeted GFP to the Golgi complex. Targeting of GFP to ER required C-terminal 66 amino acids of TC48 (350-415) which showed interaction with p25 and p23. Thus the data presented here demonstrate that TC48 translocates to Golgi complex along the secretory pathway while its ER-localization is maintained by selective retrieval enabled by interactions with p25.

454

**GAD67 is Targeted to Golgi Membranes and Presynaptic Clusters Independent of GAD65**

J. M. Kanaani (Kanaaneh), J. Do, J. M. Diacovo, S. Baekkeskov; Dept. of Medicine, Microbiology/Immunology, and Diabetes Center, University of California San Francisco, San Francisco, CA

The functional divergence between the two isoforms of the GABA-synthesizing enzyme glutamic acid decarboxylase, GAD65 and GAD67, has been attributed to differences in their subcellular localization. While GAD65 is targeted to the membrane of synaptic vesicles that secrete its product GABA, GAD67 has been reported to be a soluble cytosolic molecule that can only acquire membrane association by heterodimerization with GAD65. In this study, we have used high-resolution confocal microscopy to further analyze the subcellular localization of endogenous rat GAD67 in primary hippocampal neurons and of transfected recombinant rat, mouse, or human GAD67 or GAD67-GFP in neurons and non-neuronal cells. The analyses revealed that endogenous GAD67 in rat neurons is localized primarily in presynaptic clusters and in the Golgi compartment with a localization that is indistinguishable from endogenous GAD65. To assess whether the membrane localization of GAD67 is dependent on GAD65, primary rat hippocampal neurons were singly transfected with either rat or mouse GAD67-GFP and then immunolabeled for endogenous GAD65. In these experiments, mouse GAD67-GFP showed the same Golgi localization and distribution in presynaptic nerve terminals in neurons that lacked endogenous GAD65. In contrast, rat GAD67-GFP showed a diffuse cytosolic localization and failed to associate with membranes in the absence of GAD65. These results are consistent with the confocal immunofluorescence analysis of MDCK and COS-7 cells, where the human GAD67 and mouse GAD67-GFP were localized in Golgi membranes, while the rat protein was cytosolic. Our results suggest that membrane anchoring and subcellular localization of mouse and human recombinant GAD67 is an intrinsic property of this isoform and not dependent on GAD65. We propose that published reports describing soluble GAD67 may be attributed to a defective rat GAD67 cDNA clone.

455

**Cell-Cycle Regulation of Organelle Biogenesis and Protein Trafficking in *Toxoplasma gondii***

M. Nishi,[1] K. Hu,[2] J. M. Murray,[3] D. S. Roos[1]; [1]Biology, University of Pennsylvania, Philadelphia, PA, [2]Cell Biology, The Scripps Research Institute, La Jolla, CA, [3]Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

Apicomplexan parasites employ an unusual mechanism for replication, assembling daughters within the mother. Because rapid multiplication correlates with pathogenesis, it is important to understand the coordination of parasite assembly. With only a single copy of many subcellular organelles (unlike most eukaryotes), organellar segregation is critical to this process. Time-lapse microscopy shows that Golgi division and apicoplast (a secondary endosymbiotic plastid) elongation are among the first morphologically observable events, preceding formation of the daughter cytoskeletal scaffold. These events are associated with centriole migration from the apical to basal end of the nucleus, followed by centriolar replication, and migration back to the apical end. Cytoskeleton growth proceeds from the apical end, first encapsulating the ER/Golgi, from which the secretory organelles (micronemes and rhoptries) are produced *de novo*. Further growth of the cytoskeletal scaffold results in partitioning of the apicoplast, nucleus, and ultimately the mitochondrion, which enters the developing daughters rapidly, very late during mitosis. Defining an accurate timetable for organellokinesis permits detailed analysis of nuclear-encoded protein trafficking to apicoplast and secretory organelles. Fluorescence recovery after photobleaching (FRAP) shows that proteins target to the apicoplast via vesicles directly from ER only when the organelle is elongating prior to daughter cell formation (most efficiently during Golgi fission), but not during subsequent stages of cell division or interphase. Treatment with Brefeldin A (BFA) causes apicoplast lumenal or membrane proteins to accumulate in the ER or the tubulo-vesicular Golgi respectively only during the period of apicoplast protein trafficking. In contrast, FRAP shows that protein trafficking to micronemes transiting ER and Golgi occurs during the interphase and late cell division stage, but not during the period of efficient apicoplast protein trafficking. Treatment with BFA causes the cell-cycle dependent accumulation of microneme proteins in ER.

456

**The Role of Ent3p and Ent5p in Intracellular Trafficking in Yeast**

A. Copic, R. Schekman; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

The phosphoinositide-binding proteins Ent3p and Ent5p are required for transport of proteins from the *trans*-Golgi network (TGN) to the vacuole in *Saccharomyces cerevisiae,* but their precise role in protein sorting is not understood. Both proteins interact with the monomeric clathrin adaptor Gga2p, which is involved in trafficking to the vacuole, but Ent5p also interacts with the clathrin adaptor protein complex 1 (AP-1) *in vivo* (1). AP-1 facilitates trafficking of proteins, such as the chitin synthase Chs3p, between the early endosome and the TGN. We examined the effect of *ent3* and *ent5* mutations on the trafficking of Chs3p. Chs3p functions at the cell surface, but it is also maintained in an intracellular pool by cycling between the TGN and the early endosome in AP-1 vesicles. Chs6p is required for normal transport of Chs3p to the cell surface, but in *chs6Δ* cells, transport of Chs3p to the plasma membrane is restored by mutations in the AP-1 subunits (2). Similarly, we find that Chs3p is present at the cell surface of *ent3Δ ent5Δ chs6Δ* cells, but it is only intracellular in *ent3Δ chs6Δ* or *ent5Δ chs6Δ* cells. We isolated endogenous Ent3p and Ent5p from yeast by tandem-affinity purification, and Chs3p was found to copurify with both proteins, suggesting that Ent3p and Ent5p may directly interact with Chs3p. This interaction did not depend on the presence of the AP-1 complex. (1) Duncan, M., *et al.* (2003) *Nature Cell Biol* 5, 77. (2) Valdivia, R. H., *et al.* (2002) *Dev Cell* 2, 283.

457

**The Signal and Intracellular Location of AP-1-Dependent Sorting of Chitin Synthase III in Yeast**

T. Starr, R. Schekman; University of California, Berkeley, Berkeley, CA

In *Saccharomyce cerevisiae* the integral membrane protein chitin synthase III (Chs3p) exhibits a steady state localization at the *trans*-Golgi network and early endosomes, as well as at the bud neck region of the plasma membrane. Traffic of Chs3p to the bud neck is mediated by the peripheral membrane proteins Chs5p and Chs6p. Deletion of either Chs5p or Chs6p inhibits Chs3p trafficking to the bud neck, and thus restricts Chs3p to the *trans*- Golgi network and early endosomes. Cyclic trafficking of Chs3p between these intracellular compartments is dependent on the adaptor protein complex 1 (AP-1) (1). In a *chs6Δ* background deletion of any of the four subunits of AP-1 inhibits the trafficking of Chs3p between the *trans*-Golgi network and early endosomes, and restores Chs3p trafficking to the plasma membrane. We have identified a sorting signal in Chs3p that is required for its intracellular trafficking. The signal conforms to the types of signals to which AP-1 is known to bind, and mutation of the signal permits the trafficking of Chs3p to the plasma membrane in a *chs6Δ* background, thus mimicking the effects of AP-1 mutations. Additionally we have found that Chs3p and AP-1 physically interact and that mutations in the sorting signal impair this interaction. Therefore, we propose that we have identified an AP-1-dependent sorting signal in Chs3p. To determine the compartment at which Chs3p and AP-1 interact we are currently using microscopy to assay for the localization of fluorescently tagged Chs3p and AP-1 proteins. 1. Valdivia et al. (2002) Dev Cell 2, 283-294.

458

**Physiological Basis for Amino Acid Dependent Regulation of the *GAP1* permease**

A. L. Risinger, C. A. Kaiser; Biology, MIT, Cambridge, MA

The general amino acid permease, *GAP1*, of *S. cerevisiae* is responsible for the high capacity transport of all naturally occurring amino acids for use as a nitrogen source. Amino acid import through *GAP1* is tightly regulated by the quantity of amino acids available in the medium as well as the quality of the nitrogen source. We have determined that *GAP1* activity is repressed in response to elevated amino acid levels through transcriptional repression, ubiquitin-mediated sorting of the permease to the vacuole, and reversible inactivation of the permease at the plasma membrane. The identification of mutants that override different phases of *GAP1* regulation has allowed us to determine a physiological basis for the stringent control of *GAP1* activity. We have found that the dual transcriptional control of *GAP1* as a function of both nitrogen source quality and amino acid quantity limits expression of the permease when it would not be sorted to the plasma membrane while allowing internal pools of *GAP1* to accumulate in preparation for worsening nutritional conditions. We also find that mutants defective in the ubiquitin-mediated sorting of *GAP1* to the vacuole are sensitive to the addition of amino acids as a consequence of a toxic internal amino acid imbalance. Together, these data demonstrate that *GAP1* activity is tightly regulated in at least three distinct ways to allow for rapid changes in amino acid import to optimize growth in under a wide range of extracellular amino acid concentrations.

459

**Silencing of m1b in MDCK Cells Affects Recycling but Not Biosynthetic Delivery of TfR**

D. Gravotta,[1] A. Deora,[1] R. Schreiner,[1] A. Soza,[2] C. Oyanedel,[2] A. González,[2] E. Rodriguez-Boulan[1]; [1]Dyson Vision Research Institute, Weill Medical College of Cornell University, New York, NY, [2]Depto. Immunol. Clin. and Reumatol., Fac. Medicina and Centro de Regulación Celular y Patología, Fac. Ciencias Biológicas. Pontificia Universidad Católica de Chile, and MIFAB, Santiago, Chile

To date, all studies on the sorting role of the epithelial adaptor AP1B have been carried out in LLC-PK1 cells, a pig epithelial cell line, which naturally lacks m1B subunit and sorts LDLR and TfR to the apical plasma membrane. Here, we have silenced the expression of dog m1B by transfection of 21mer- double stranded RNA oligonucleotides or pSuper vector carrying silencing sequences. MDCK cells transiently and permanently silenced for m1B (m1B-KD MDCK) distributed LDLR and TfR to the apical membrane. We then used a novel biochemical approach involving pulse chase and the addition of apical or basolateral biotinylated Tf to study the biosynthetic and recycling routes of Tf-R in polarized m1B-KD MDCK cells. Biosynthetic delivery of Tf-R occurred as in control cells, preferentially towards the basolateral membrane. In contrast, recycling of TfR occurred preferentially towards the apical membrane. We conclude that, as previously shown in LLC-PK1 cells (Gan, McGraw and Rodriguez-Boulan, Nature Cell Biology, 2002), the sorting function of AP1B appears to be in a post-endocytic compartment; this recycling compartment does not appear to be located in the biosynthetic route of TfR. Supported by grants from NIH to ERB and by FONDAP and Millenium grants to AG.

460

**Two Step Targeting of AAM-B to Lipid Droplets (Adiposomes)**

J. K. Zehmer, R. Bartz, P. Liu, R. G. W. Anderson; Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX

Recently our knowledge of lipid droplet (adiposome) proteins has expanded dramatically due to several proteomic analyses in several cell types. However, our understanding of how these proteins are targeted to adiposomes is still very limited. Analyses of adipophilin and perilipin found that these proteins have multiple, redundant regions containing targeting information, but a specific targeting signal has not been identified. We previously found the protein AAM-B (also known as DKFZp586A0522) in a proteomic analysis of CHO K2 adiposomes. In the current study we have examined in detail the targeting of this putative methyltransferase to HeLa cell adiposomes by transient transfection and immunofluorescence of immuno-tagged constructs. We found that AAM-B is directed to adiposomes by two distinct signals. First, an N-terminal hydrophobic alpha helix directs AAM-B to the endoplasmic reticulum. There, a second signal, contained in a 45 amino acid region just C-terminal to the hydrophobic helix, directs AAM-B to the adiposome. An alanine scan of this region identified two 3 amino acid patches that are critical for targeting. The hydrophobic helix and the downstream region, contained in amino acids 1-73, are sufficient to target GFP to adiposomes. Finally, we demonstrate that AAM-B forms homo-dimers or possibly higher-order oligomers, but that oligomerization is not required for adiposome localization.

461

**Ykt6 and Sedlin Colocalize in a Novel Cytoplasmic Protein-Lipid Particle**

Y. Liang,[1] H. Hasegawa,[2] V. Oorschot,[3] J. Klumperman,[3] J. C. Hay[1]; [1]Division of Biological Sciences, University of Montana, Missoula, MT,

[2]National Institutes of Health, Bethesda, MD, [3]Department of Cell Biology, University Medical Center Utrecht, Utrecht, The Netherlands
The function and targeting of the mammalian SNARE ykt6 has been controversial. While others have reported a Golgi localization and transport function in kidney cell lines, we reported that ykt6 is expressed primarily in neurons, where its longin domain targets the protein to unidentified particulate structures assumed to represent novel membrane organelles. Here we used immunoelectron microscopy to examine the novel structures in PC12 cells. They appeared as ~200 nm electron-dense particles lacking a limiting membrane and exhibiting ykt6 labeling throughout. These particles precede cell fixation and represent the "natural" state of ykt6. To reconcile differing views of ykt6, we quantified its participation in traditional membrane SNARE complexes versus non-traditional organelles. Up to 20% of particulate ykt6 in PC12 cells can participate in SNARE complexes, while ~80% appears to be unavailable and resides within a protease-resistant structure presumably corresponding to the electron-dense particles. The protease-resistant structures lack a chaotropic agent-protective membrane but their buoyant density indicated that they contained significant lipid. Although ykt6 can be doubly lipidated, the particles did not appear to represent ykt6 "micelles" whose association is driven by lipid interactions. Rather, the particles appear to originate by self-association of longin domains, with the lipid, not necessarily covalently attached to ykt6, sequestered in the lipid-binding surfaces of the longin domain. Finally, we found that ykt6 was not the only longin protein present in the particles. Sedlin, mutations in which cause the skeletal disease *spondiloepiphyseal dysplasia tarda*, co-localized with ykt6 to the structures. In summary, ykt6 and sedlin exist naturally in ~200 nm, protease-resistant, protein-lipid particles not related to traditional SNARE function that have the potential to act as cytoplasmic "lipid rafts". We speculate that these longin structures may be important for neuronal lipid storage, transport, or metabolism.

462

**Stimulation of G-protein Coupled Receptors Induces Nuclear Localization and Membrane Translocation by Palmitoylation of Histamine N-Methyltransferase**

M. Ogasawara,[1] K. Yamauchi,[2] Y. Satoh,[3] K. Maeyama[1]; [1]Depart. of Integrated life science Div. of Pharmacology, Ehime University School of Medicine, Toon city, Japan, [2]Third Depart. of Medicine, Iwate Medical University, Morioka city, Japan, [3]Depart. of Anatomy, Iwate Medical University, Morioka city, Japan
Objects: Histamine *N*-methyltransferase (HMT) is a principal enzyme to inactivate histamine, localizes in airway epithelium and plays a critical role in regulating allergic reaction of histamine. We reported the translocation of HMT molecules to plasma membrane with plasma membrane protein biotinylation method. However, the molecular mechanism of translocation remains elucidated. Using endogenously expressed histamine receptor and β adrenergic receptor in HEK293 cells, we investigated the molecular basis of translocation of HMT. Methods: HEK293 cells stably transformed with C-terminal HA tagged HMT cDNA were cultured for 4 hours with [$^3$H] palmitic acid in the presence or absence of isoproterenol or/and histamine. Cell extracts were immunoprecipitated with antibodies against HA tag. Incorporation of [$^3$H] palmitic acid were measured. Moreover, the HEK293 cells were stimulated in the presence or absence of beta adrenergic receptor agonist, isoproterenol (10υM) and/or histamine followed by staining with antibodies against HA tag and immunofluorescein. Results: HMT molecules were palmitoylated and inhibited by palmitoylation inhibitor, 2-bromopalmitic acid. Beta adrenergic receptor stimulation enhanced the palmitoylation of HMT. Moreover the stimulation of beta adrenergic receptor and /or histamine receptor induced the nuclear translocation of HMT. Conclusion: HMT molecules were modified with palmitoylation, which was enhanced with stimulation of beta adrenergic receptor, suggesting that molecular basis of plasma membrane localization of HMT can be partly explained by lipid modification. Translocation of HMT from cytoplasm to nucleus after stimulation with beta adrenergic receptor and/or histamine receptor suggest that HMT may have other function than histamine inactivation or another function of histamine in the nucleus could be suggested because of histamine transportation into cell through organic cation transporters. Moreover we may need to reevaluate the substrate specificity of HMT for amine derived compounds.

463

**The Phogrin Luminal Domain Dense Core Granule Sorting Signal Can Act at the Plasma Membrane**

J. Wenzlau,[1] P. Burgos,[1] R. Bauer,[1] C. Wasmeier,[2] H. Davidson,[1] J. Hutton[1]; [1]Barbara Davis Center, UCDHSC, Aurora, CO, [2]Imperial College, London, United Kingdom
Phogrin is an integral membrane protein of neuroendocrine dense-core secretory granules (SGs). Newly synthesized phogrin apparently traffics to SGs without prior exposure at the plasma membrane (PM). Previously we have shown that key targeting information resides in both the luminal and cytoplasmic domains. Mutants lacking the cytosolic domain (ΔC), and a chimera comprising the luminal and transmembrane domains of Tac fused to the phogrin cytoplasmic domain, are both efficiently sorted to SGs in transfected AtT20 cells. However, it is uncertain if either mutant follows an identical intracellular itinerary to the native molecule. To address this question we have studied in more detail the trafficking and post-translational processing of wild-type and mutant phogrin. Pulse-chase experiments show that both wild-type phogrin (WT; 130kDa) and ΔC (100kDa) are proteolytically processed to their mature forms in an acidic post-TGN compartment within 2h of labelling. Processed ΔC co-distributes with both processed mature WT and insulin on sucrose density gradients, suggesting that proteolysis occurs in immature and/or mature SGs. In contrast to WT, a significant fraction of radiolabelled ΔC-precursors can be surface biotinylated, indicative of their mis-sorting to the PM. However, biotinylated ΔC-precursors are also efficiently processed, suggesting that this mutant can follow an indirect route to SGs. Antibody uptake experiments demonstrate that mature WT is internalized after stimulated secretion, and within 30 min accumulates in perinuclear structures that co-localize with internalized transferrin. Phogrin ΔC precursors similarly congregate in transferrin positive perinuclear compartments implicating recycling endosomes in post-endocytic transit to SGs. We conclude that signals in the luminal domain of phogrin can act at the PM and/or transferrin positive endosomes, rather than exclusively at the TGN as we had previously proposed.

464

**The Transmembrane Domain of the PC3: A Key Motif for Targeting to the Regulated Secretory Pathway**

H. Lou,[1] A. M. Smith,[2] L. C. Coates,[2] N. P. Birch,[2] Y. P. Loh[1]; [1]Section on Cellular Neurobiology, NICHD/National Institutes of Health, Bethesda, MD, [2]Laboratory of Molecular Neuroendocrinology, University of Auckland, Auckland, New Zealand
The biosynthesis of hormones and neurotransmitters involves post-translational cleavage of proprotein precursors at basic amino acid cleavage sites by a family of prohormone convertases. Processing of precursors in regulated secretory pathway occurs primarily in secretory granules that bud

from the *trans* Golgi Network. This study reports a role for the transmembrane domain (aa 617-638) of the prohormone convertase 3 (PC3) in sorting to regulated secretory granules. Membrane extraction and sucrose density gradient analyses showed differential association of full length (PC3-FL) and carboxyl terminus-truncated forms of PC3 with (PC3-638) or without (PC3-616) the transmembrane domain in Neuro2A cell membranes. Similar to PC3-FL, PC3-638 was associated with lipid rafts in membranes but not PC3-616. PC3-638 showed robust stimulated secretion. Immunocytochemical analysis demonstrated a high level of co-localization of PC3-638 with a secretory granule marker, chromogranin A, in cell processes. In contrast, PC3-616 underwent constitutive secretion and was localized mainly in the perinuclear region of the cell body. These results indicate that within the framework of the PC3 molecule, the transmembrane domain ($PC3_{619-638}$) plays a key role in sorting the enzyme to the regulated secretory pathway. We therefore propose that interaction of the transmembrane sorting domain of PC3 with lipid rafts at the *trans* Golgi Network is the mechanism by which PC3 is sorted into the budding granules of the regulated secretory pathway.

465

**Determining the Subcellular Localization of Type II Membrane Proteins**

R. N. Aturaliya,[1] L. Fink,[1] M. J. Davis,[1] M. S. Teasdale,[1] K. A. Hanson,[1] K. C. Miranda,[1] H. Suzuki,[2] R. D. Teasdale[1]; [1]Institute of Molecular Biology, Brisbane, Australia, [2]RIKEN Genomic Sciences Centre, Yokohama, Japan

Subcellular localization plays a pivotal role in determining protein function. The subcellular localization of membrane proteins is dependant on features such as signal peptides and transmembrane domains which determine membrane organization. A computational membrane organization prediction pipeline, MemO, classifies putative type II membrane proteins as proteins predicted to encode a single alpha-helical transmembrane domain and which lack a predicted signal peptide. MemO was applied to RIKEN's Isoform Protein Set which identified 2,869 transcripts encoding putative type II membrane proteins clustered into 2,149 non-overlapping genomic regions, or Transcriptional Units (TUs). This study focuses on TUs containing exclusively type II transcripts. Transcripts with overlapping InterPro and transmembrane domains were reviewed to remove proteins with inaccurate transmembrane domain predictions. Using this dataset, we developed a systematic protocol to document the subcellular localization of type II membrane proteins. This approach combines mining of published literature to identify subcellular localization data as well as a high-throughput, PCR-based experimental approach to characterize subcellular localization. These approaches have provided localization data for 221 and 169 proteins, respectively. Type II membrane proteins localize to all major organelle compartments, however, some biases were observed including a high prevalence in the early secretory pathway and punctate structures. Collectively, this study reports the subcellular localization of 24% of the final type II dataset comprised of 1,407 TUs. Localization data for all classes of membrane proteins is presented in a web-accessible database, LOCATE (http://locate.imb.uq.edu.au). Currently, the database reports membrane organization predictions as well as literature-mined and experimentally determined high-throughput subcellular localization data. The database contains literature localization data for over 1400 TUs, of which over 1000 represent membrane proteins, and experimental localization data for over 400 membrane proteins.

466

**Differential Targeting of R9AP and Syntaxin 3 in Transgenic *Xenopus* Photoreceptors**

S. A. Baker,[1] M. Haeri,[2] B. E. Knox,[2] V. Y. Arshavsky[1]; [1]Ophthalmology, Duke University Medical Center, Durham, NC, [2]Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, NY

Photoreceptors are compartmentalized, polarized sensory neurons. At the distal end of the cell is the outer segment, a ciliary organelle specialized in phototransduction. At the proximal end of the cell is the synaptic terminal which communicates with the secondary neurons of the retina. The mechanisms that specifically target proteins to these different compartments are largely unknown. R9AP (RGS9 Anchor Protein) is located in the outer segment and is a member of the extended SNARE protein family. It is required for the delivery of the GTPase activating protein, RGS9 (Regulator of G protein Signaling, family member 9), to the outer segment and for the regulation of its activity. The structural organization of R9AP is identical to that of syntaxin 3 which is the major syntaxin protein in photoreceptor synaptic terminals. Both contain two cytoplasmic domains, a three helical bundle and the SNARE homology domain, followed by a transmembrane domain at the C-terminus. The goal of this study was to identify the domains responsible for the differential targeting of these proteins. Our approach was to transgenically express EGFP-tagged mutants of these proteins in *Xenopus* rod photoreceptors. We have found that C-terminally tagged full-length R9AP and syntaxin 3 are correctly targeted to their respective subcellular locations. Minimal constructs containing just the transmembrane domain of either protein was found to accumulate in the outer segment. We conclude that in photoreceptors the targeting motif is located in the cytoplasmic parts of these proteins. This is in contrast to the finding in polarized epithelial cells where the transmembrane domains of syntaxins 3 and 4 do mediate their apical versus basolateral membrane targeting (Bulbarelli et.al, 2002). We are now using chimeric R9AP/syntaxin 3 proteins to identify the specific location of the targeting motif used in photoreceptors.

467

**Development of a Novel, Raman-Active Labeling System for Real-Time Monitoring of Cellular Processes**

W. E. Doering, M. Sha, M. Yamanaka, G. Freeman, S. Penn, M. J. Natan; Biotechnology, Nanoplex Technologies, Inc., Mountain View, CA

Imaging of biochemical functionality within live cells can occur only with the use of an appropriate biological stain or dye to generate the contrast needed for image generation. The ability to image simultaneously multiple different functions therefore requires multiple distinct detection tags. Techniques have been traditionally limited to the simultaneous detection of just a few species, due to the limited excitation and emission profiles of organic fluorophores. Recently, quantum dots have shown the potential to expand the multiplexing capability of optical techniques to perhaps five colors. We have recently developed a completely novel optical tag based on surface-enhanced Raman scattering (SERS). The tags are composed of a gold core that is coated with a Raman "reporter" molecule and encapsulated with a silica shell. Upon excitation, the profiles of the tags are determined exclusively by the Raman reporter. SERS nanotags have many optical properties that make them ideal candidates for cellular imaging; they can be easily excited in the near-IR (785 nm for example), they do not photobleach (allowing for extended viewing experiments), and distinct optical signatures are easily generated without changing the properties of the nanoparticles, such as size or surface chemistry. We have selectively conjugated two distinct "flavors" of SERS nanotags to separate antibodies and labeled SKBR-3 breast cancer cells based on the expression of the her2-neu receptor and a nuclear envelope protein. We have also developed efficient optical tags producing 4 distinct spectral responses upon NIR excitation, which is highly favorable to the UV-excitation required to monitor multiple quantum dot flavors. It is straightforward to envision

expansion to 10-20 different flavors of SERS-active tags, which would allow unprecedented real-time tracking of multiple proteins and pathways in cells under NIR excitation.

468

**Enzyme Recruitment and the Distributions of 3' Phosphoinositides during Fc Receptor-Mediated Phagocytosis**

L. A. Kamen, J. A. Swanson; Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI

Enzyme Recruitment and the Distributions of 3'Phosphoinositides during Fc Receptor-Mediated Phagocytosis Changing concentrations of 3' phosphoinositides at the plasma membrane define signaling transitions that allow Fc receptor-mediated phagocytosis in macrophages. Phosphatidylinositol 3 kinase (PI3K) and the lipid phosphatases PTEN and SHIP-1 are capable of modifying levels of 3'phosphoinositides and are important in this process. It was previously shown that both SHIP-1 and p85, the regulatory subunit of type I PI3K, localize to the phagosome. Moreover, 3' phosphoinositides have been localized to the phagosomes, but their dynamics relative to enzyme localization are not well defined. We used ratiometric fluorescence microscopy of YFP chimeras expressed in macrophages to examine the dynamics of phosphoinositides and the enzymes that control their concentrations during FcR-mediated phagocytosis. The distribution of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ were inferred using YFP chimeras of pleckstrin homology (PH) domains from Plcδ1 and Btk, respectively. $PI(4,5)P_2$ was present initially on the plasma membrane but diminished throughout the course of phagocytosis. In contrast, $PI(3,4,5)P_3$ was absent from resting plasma membrane, increased on the phagosome and remained elevated until after closure. YFP-p85 was present on the phagosome throughout phagocytosis and remained after closure in punctate structures. YFP-PTEN and YFP-SHIP-1 showed different distributions during phagocytosis; YFP-PTEN never localized to the phagosome but YFP-SHIP-1 localized to the phagosome at the initiation of phagocytosis and redistributed to the cytosol after closure. Both probes slowed phagocytosis. These results indicate that $PI(3,4,5)P_3$ is the essential 3' phosphoinositide in FcR-mediated phagocytosis. Its levels are regulated by the exclusion of PTEN and the simultaneous recruitment of PI3K and SHIP-1 to the phagosome. These results further indicate that the changing profiles of 3' phosphoinositides on phagosomal membranes are regulated by mechanisms for enzyme deactivation, rather than by sequential enzyme recruitment.

469

**Targeting of the Small GTPase ARL10c to the Lysosomal Membrane**

I. M. Hofmann, S. Munro; Cell Biology, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

ARF-like proteins (ARLs) are small GTPases with homology to ADP-ribosylation factors (ARFs). Whereas ARFs are important regulators of membrane traffic, ARLs seem to be involved in processes as diverse as vesicle transport (ARL1) and tubulin folding (ARL2). To examine the function of the human ARLs more closely, we started investigating their intracellular localization. ARL1, ARL5 and ARL8 localize to the Golgi, ARL4, ARL4L and ARL7 to the plasma membrane and ARL10c to lysosomes. ARL10c is targeted to the lysosomal membrane by a mechanism unusual for the ARL-proteins. Conventionally, membrane localization of ARF- and ARL-proteins is conferred by both an N-terminal myristate and an N-terminal amphipathic helix, which insert into the lipid bilayer. Instead of the N-terminal myristate ARL10c is N-terminally acetylated, and this acetyl-group is necessary for its lysosomal localization. In addition, mutation of hydrophobic residues in the amphipathic helix of ARL10c abolishes membrane localization of ARL10c. This indicates a function of the N-terminal helix in membrane attachment similar to other ARFs/ARLs. Thus, ARL10c is targeted to the lysosomal membrane by both an N-terminal acetyl-group and the amphipathic helix. Overexpression of ARL10c results in a redistribution of lysosomes towards the cell periphery. Furthermore, ARL10c seems to control lysosomal transport by increasing frequency and run length of transport events and by decreasing pauses and small movements. This suggests a role for ARL10c as positive regulator of lysosomal transport.

470

**Autocrine Motility Factor Receptor, a Ubiquitin E3 Ligase, is Monoubiquitylated and Regulates p97/VCP AAA ATPase ubiquitylation**

P. St-Pierre,[1] T. Dang,[1,2] I. Nabi[1]; [1]Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, [2]Département de Pathologie et de Biologie Cellulaire, Université de Montréal, Montréal, PQ, Canada

Activation of autocrine motility factor receptor (AMFR) by its ligand, AMF, is associated with cellular transformation and protection from apoptosis and stimulates tumor cell motility and angiogenesis. AMFR is also a multimembrane spanning ubiquitin E3 ligase involved in endoplasmic reticulum-associated degradation (ERAD) whose cytoplasmic domain contains both a RING finger and a CUE domain. The p97/VCP AAA ATPase regulates ubiquitylation and proteasomal targeting of substrates for ERAD and has been reported to be associated with the AMFR cytoplasmic tail. Here we report two monoubiquitylated forms of AMFR whose expression is dependent on the integrity of the RING finger domain. In contrast, disruption by point mutations of the CUE domain function does not significantly impair monoubiquitylation of AMFR. p97 coimmunoprecipitates with AMFR and exhibits partial colocalisation to the smooth endoplasmic reticulum with AMFR. Expression of AMFR promotes monoubiquitylation of various substrates including p97. AMFR-p97 interaction is not affected by disruption by point mutation of either the RING or Cue domains while monoubiquitylation of p97 is RING finger but not Cue domain dependent. The RING finger domain of AMFR is therefore implicated in monoubiquitylation of both AMFR and p97. Supported by the CIHR.

471

**Delta Aminolevulinic Acid Dehydratase Regulates Proteasome Activity**

F. Bardag-Gorce,[1] A. Banerjee,[2] J. Li,[1] B. A. French,[1] S. W. French[3]; [1]Pathology, LABiomed at Harbor-UCLA Med Center, Torrance, CA, [2]Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI, [3]Pathology, Harbor-UCLA Med Center, Torrance, CA

The proteasome interacts with a large number of proteins in order to perform specific functions. The focus of this study is to examine the proteasome interaction with Delta aminolevulinic acid dehydratase (ALAD), which is involved in the heme biosynthesis pathway. This enzyme interacted and was co-purified with the 20S proteasome using several chromatographic purification steps. Tryptic digestion was used followed by ESI mass spectrometry analysis to identify this 20S interacting protein as ALAD. When the 26S proteasome was isolated using density-gradient centrifugation, ALAD stained positive in Western blot of the 26S proteasome fraction. This result indicated that ALAD interacts with the proteasome. However proteasome inhibition in vivo achieved by giving PS-341 to the rats, did not change the ALAD protein level in liver

homogenates. When mice are fed diethyl 1,4-dihydro-2, 4,6 trimethyl-3,5-pyridine dicarboxylate (DDC), they develop protoporphyria due to the disruption of heme synthesis. In this model the ALAD protein level was decreased and the proteasome beta5 subunit (subunit carrier of the chymotrypsin-like activity) level was up regulated. This data supports the hypothesis that ALAD, as an important enzyme for heme synthesis, is also important as a proteasome modulator, which is consistent with the double function of the ALAD protein. Thus ALAD may function as an important regulator of proteasomal activity. Supported by NIH/NIAAA Grants 8116 and GM-59467.

472

**Evidence for Metabolic Coupling of Peroxisomes and Lipid Bodies**

D. Binns,[1] T. Januszewski,[2] Y. Chen,[3] Y. Zhao,[3] C. Gilpin,[2] K. Chapman,[4] J. M. Goodman[1]; [1]Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, [2]Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX, [3]Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, [4]Biological Sciences, University of North Texas, Denton, TX

We and others have observed close physical interactions between peroxisomes and lipid bodies. In the yeast Saccharomyces cerevisiae growing on oleic acid (a condition in which both peroxisomal and lipid body metabolism is induced) fluorescence microscopy shows that most peroxisomes associate with lipid bodies. Thin section electron microscopy reveals that peroxisomes wrap around lipid bodies and even extend processes into their cores. Purified lipid bodies are enriched in peroxisomal enzymes that catalyze fatty acid beta-oxidation, but not other peroxisomal proteins, suggesting that "peroxisomal" beta oxidation is tightly coupled to the metabolism of lipid bodies, a source of fatty acids. We describe novel morphological structures within lipid bodies we term "gnarls",that may represent accumulations of fatty acids. Gnarls are particularly abundant in strains containing peroxisomes that are unable to metabolize fatty acids, yet they are less developed in a strain entirely lacking in peroxisomes. We propose that peroxisomal contact with lipid bodies stimulates the generation of fatty acids at the lipid body, which are then utilized by peroxisomal enzymes embedded in the lipid body or adjacent to it. If peroxisomes are defective, fatty acids accumulate to create the gnarls.

473

**The Peroxisomal Membrane Protein, Pex16p, is Involved in the Formation Peroxisomes at the Endoplasmic Reticulum**

P. K. Kim,[1] R. T. Mullen,[2] J. Lippincott-Schwartz[1]; [1]Cell Biology & Molecular Biology, NICHD/NIH, Bethesda, MD, [2]Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Peroxisomes are small, membrane-bound organelles present in almost all eukaryotic cells. Although defects in peroxisome biogenesis are involved in various diseases, the molecular mechanisms involved in peroxisome biogenesis are poorly understood. In yeast, plants, and mammals, evidence exists for both fission and *de-novo* formation of peroxisomes. However, whether the *de-novo* assembly of peroxisomes is dependent on the endoplasmic reticulum (ER) or some other endomembrane system is still a matter of debate. To examine the possible role of the ER in peroxisome biogenesis, the cellular localization of Pex16p (a peroxisomal membrane protein/peroxin essential for peroxisome biogenesis) was examined using cell-free targeting assays and confocal microscopy techniques with green fluorescence protein (GFP) chimeras. We show that Pex16p is co-translationally targeted to the ER *in vitro*, in contrast to the reported post-translational targeting of other peroxisomal proteins. We show also that a Pex16-GFP fusion protein localizes to both ER and peroxisomes in COS7 cells, but only to ER in *pex19* mutant cells, which lack peroxisomes. A modified version of Pex16-GFP fusion protein containing a *bona fide* ER targeting signal sequence at its amino-terminus also localizes to both ER and peroxisome in COS7 cells, and can functionally complement *pex16* mutant cells. These data suggest that the ER-targeted version of Pex16-GFP is sufficient for the production of new peroxisomes and that the machinery responsible for peroxisomal import is derived from the ER. Consistent with this premise, time-lapse imaging of live cells revealed that a Pex16-Photoactivatable-GFP fusion protein that was photoactivated while in the ER was localized eventually to peroxisomes. Overall, these data demonstrate the ER origin of mammalian Pex16p and support an emerging model for peroxisome biogenesis in evolutionary diverse organisms wherein the organelle forms *de-novo* from the ER.

474

**Cloning and Characterization of Peroxisomal Membrane Protein-70 Throughout *Xenopus laevis* Development**

C. A. Cooper,[1] P. A. Walton,[2] S. Damjanovski[1]; [1]Biology, University of Western Ontario, London, ON, Canada, [2]Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada

Peroxisomes are ubiquitous single membrane bound organelles, which are essential for eukaryotic cell function. They facilitate the chemical breakdown of fatty acids through the process of β-oxidation. Peroxisomal membrane proteins (PMPs) are of particular interest as defects in these proteins result in improper peroxisome function. Peroxisomes have been characterized in several organs and tissues and play important roles in cell senescence. Little is known however, about their role in development. Furthermore, there is no research to date concerning how peroxisomes are associated with the complex physiological changes that occur during frog metamorphosis. In this study, *Xenopus* PMP-70 was cloned and characterized and its spatial and temporal expression pattern analyzed throughout early development and metamorphosis. The amino acid sequence of *Xenopus* PMP-70 shows 92% similarity to mammalian PMP-70. Embryo microinjections of a tagged GFP directed to peroxisomes resulted in the localization of GFP in aggresomes prior to embryonic stage 22. This suggests a lack of functional peroxisomes in early development, a finding confirmed by RT-PCR and whole mount *in situ* analysis. PMP-70 was predominantly localized following stage 34 to the developing eye, pharyngeal arches, and dorsal neural tissues. This finding is consistent with semi quantitative RT-PCR which shows a steady increase in PMP-70 RNA throughout development. Interestingly, PMP-70 RNA levels remain relatively constant during the metamorphosis of the heart, limb and tail. In the liver however, PMP-70 RNA increases dramatically at the peak of metamorphosis. This is at a time when fatty acid stores are being metabolized at an increased rate as tadpoles undergoing metamorphosis do not take in food. Although peroxisomes continue to be implicated in cell senescence, these findings suggest important roles for peroxisomes during the development of specific tissues and organs.

475

**Intracellular Redistribution of Catalase Provides Increased Cellular Protection Against Non-peroxisomal Oxidative Stress**

A. Pellicoro, F. A. J. van den Heuvel, K. Landman, K. N. Faber; Gastroenterology and Hepatology, UMCG, Groningen, The Netherlands

Background & aims: Catalase is a peroxisomal enzyme that detoxifies $H_2O_2$ produced by peroxisomal oxidases. Catalase is also regarded as an antioxidant enzyme that protects cells against non-peroxisomal oxidative stress. Human cells are continuously exposed to oxidative stress and there

is ample evidence that reactive oxygen species are a key factor in development of aging. Moreover, oxidative stress is an important contributor to the pathology of a variety of diseases. Catalase may redistribute to the cytosol in aging cells or when cells are exposed to $H_2O_2$. We determined the effect of intracellular redistribution of catalase on protection of cells against exposure to $H_2O_2$. Methods: Cells from the human hepatoma cell line HepG2 were grown in the presence of variable amounts of $H_2O_2$, with or without the addition of the catalase inhibitor 3-aminotriazol (3-AT). Sytox green was used to visualize (by fluorescence microscopy) and quantify (by fluorometry) necrosis. HepG2-derivative cell lines were generated that overexpress peroxisomal or nuclear catalase. RNA interference (RNAi) was used to 1) decrease expression of catalase or 2) disrupt peroxisomal targeting of catalase by reducing expression of the signal receptor, Pex5p. Real time RT-PCR, Westernblot analyses and immunohistochemistry were used to determine mRNA levels, protein levels or the subcellular location of catalase, respectively. Results: 3-AT and RNAi-reduction of catalase strongly sensitized HepG2 cells for $H_2O_2$-induced necrosis. Both nuclear and peroxisomal overexpressed catalase gave significant protection against $H_2O_2$ stress. The nuclear catalase was more protective than the native one. RNAi-reduction of Pex5 resulted in cytosolic catalase. These cells showed an increased protection against extra-peroxisomal $H_2O_2$. Conclusions: Redistribution of catalase to the cytosol provides increased cellular protection against extracellular oxidative stress. This may be a functional adaptation to face increased oxidative stress during aging or disease.

## Gene Structure and Expression (476-495)

476

### Transcriptional Regulation of the Mammalian ATP Synthase Alpha-Subunit Gene

G. A. Breen, A. Alam, R. Pittman; Molecular and Cell Biology, University of Texas at Dallas, Richardson, TX

Mitochondrial biogenesis is a highly regulated and coordinated process. Our laboratory has been studying the transcriptional regulation of the nuclear gene that encodes the α-subunit of the mammalian mitochondrial ATP synthase complex (*ATPA*). We have now identified a number of regulatory factors which regulate the expression of the *ATPA* gene. For example, we have determined that the transcription factor, upstream stimulatory factor 2 (USF2), binds to an E-box element (CANNTG) in the upstream promoter of the *ATPA* gene and stimulates expression of this gene. We have also determined that USF2 activates the *ATPA* gene through binding to an initiator element in the core promoter. Furthermore, we have determined that the transcriptional coactivator, p300, interacts functionally with USF2 to activate the *ATPA* gene. We have also identified a negative regulatory factor, COUP-TFII/ARP-1, which represses the expression of the *ATPA* gene. Furthermore, we have determined that COUP-TFII/ARP-1 inhibits the USF2-mediated activation of the *ATPA* gene promoter. Electrophoretic mobility shift assays revealed that COUP-TFII/ARP-1 and USF2 compete for overlapping binding sites in the upstream promoter of the *ATPA* gene. Recently, we have demonstrated a role for the coactivator, PGC-1α, in the transcriptional regulation of the *ATPA* gene. It is likely that the net expression of the *ATPA* gene will result from the relative concentration and affinity of these regulatory factors (and probably other factors) expressed in a given physiological state. These factors might play important regulatory roles linking changes in expression of the *ATPA* gene with changes in cellular energy needs.

477

### Transcriptional Effect of Oxidative Stress in Normal Human Keratinocytes using Low Density cDNA Microarray

M. Bonnet-Duquennoy, K. Lazou, S. Talbourdet, S. Schnebert, R. Kurfürst; Life Sciences Dpt, LVMH Recherche, St Jean de Braye, France

Skin is a major target of oxidative stress due to reactive oxygen species (ROS) that originate in the environment and in the skin itself. ROS are generated during normal metabolism and cause oxidative alterations on cell constituents causing development of cutaneous disorders as ageing and more serious lesions as cancer. Although ROS play a crucial role on skin regulation, their biological targets and pathogenic mode of action are still not fully understood. In order to study mechanisms of oxidative stress response, we developed a sensitive, rapid and quantitative microarray method. We have chosen OLISA™ (OLIgo Sorbent Array) technique, an oligonucleotide microarrays developed by bioMérieux. The gene panel screened involved 105 skin-related genes coding for proteins associated with cell death, proliferation, anti-oxidant defense mechanisms, signal transduction, epidermal structure and differenciation. With this technique, gene expression was assessed in normal human keratinocytes at the basal level and after a hydrogen peroxide treatment (25µM). We showed that more than 85 of genes (80%) were detected in the cells studied. We observed major modification at transcriptional level between 6 and 24 hours after hydrogen peroxide treatment. Moreover, we showed that 30% of detected genes had differing expression levels. Focusing on anti-oxidant genes, 75% of detected genes were significantly regulated. Significant increases in metallothionein, thioredoxin and SOD1 expression were observed showing induction of defense mechanism by oxidative stress. A marked difference was observed in the decreased gene expression of p63 and p73, transcriptional factors involved on cell cycle regulation and keratinocyte differentiation suggesting alteration of these mechanisms by oxidative stress. In conclusion, this method associated with human keratinocyte culture provides a good strategy for understanding physiological response to oxidative stress in epidermis and studying new protective molecules.

478

### Protein Folding Information in Nucleic Acids which is Not Present in the Genetic Code

J. C. Biro; Homulus Foundation, San Francisco, CA

**Background:** All the information necessary for protein folding is supposed to be present in the amino acid sequence. It is still not possible to provide specific ab initio structure predictions by bioinformatical methods. It is suspected that additional folding information is present in protein coding nucleic acid sequences, which is not represented by the known genetic code. **Results:** Nucleic acid subsequences comprising the 1st and/or 3rd codon residues in mRNAs express significantly higher free folding energy (FFE) than the subsequence containing only the 2nd residues ($p$<0.0001, $n$=81). This periodic FFE difference is not present in introns and therefore it is a specific physico-chemical characteristic of coding sequences and it might contribute to unambiguous definition of codon boundaries during translation. The FFE in the 1st and 3rd residues is additive, which suggests that these residues contain a significant number of complementary bases and contribute to selection for local RNA secondary structures in coding regions. This periodic, codon-related structure-forming of mRNAs indicates a connection between the structure of exons and the corresponding (translated) proteins. The folding energy dot plots of RNAs and the residue contact maps of the coded proteins are indeed similar. Residue contact statistics using 81 different protein structures confirmed that amino acids that are coded by partially reverse and

complementary codons (Watson-Crick (WC) base pairs at the 1st and 3rd codon positions and translated in reverse orientation) are preferentially co-located in protein structures. **Conclusions:** The preferentially (specifically) interacting amino acids are coded by partially complementary codons, which strongly supports the connection between mRNA and the corresponding protein structures and indicates that there is protein folding information in nucleic acids that is not present in the genetic code.

479

**Characterization of the Luteinizing Hormone beta-Subunit Precursor Protein cDNA in the Rabbit**

O. Suzuki, M. Koura, Y. Noguchi, K. Takano, K. Uchio-Yamada, J. Matsuda; Laboratory of Experimental Animal Models, National Institute of Biomedical Innovation, Ibaraki-shi, Japan

To elucidate the molecular basis of efficient superovulation in the rabbit, we determined the cDNA sequences of the luteinizing hormone β-subunit (LHB) for three breeds of rabbit (Japanese White, JW; New Zealand White, NZW; and Dutch Belted, Dutch) using a combination of 5'- and 3'-rapid amplification of cDNA ends (RACE) with pituitary cDNA libraries, and compared them with those of other mammals. The RACE experiments revealed that the LHB cDNA of all three breeds were the same length (524 bp from the 5'-end to the polyA site), with a putative AATAAA polyadenylation signal sequence at positions 505 to 510. Their 426-bp-long coding sequences had a higher GC content (>73%) than that of other mammals (~65%). The deduced amino acid sequence of JW, which was 141 residues long, was exactly the same as that of NZW, but differed from that of Dutch at two positions. Phylogenetic analysis of the deduced amino acid sequences of the three rabbits' LHB and those of laboratory and domestic animals suggest that the rabbit stands in a phylogenetically intermediate position between rodents and domestic animals, as we showed in a similar analysis of FSH β-subunits at the 2004 ASCB meeting. These unique features of rabbits might be related to their unique reproductive endocrine system, copulatory ovulation. The cDNA sequence of JW LHB has been deposited in GenBank (accession number: AY614703).

480

**SUMO Modification and Acetylation Regulate the Activity and Stability of the ETS Transcription Factor, Myeloid Elf-1 like Factor**

H. Nakamura,[1] M. S. Suico,[1] Z. Lu,[1] H. Saitoh,[2] T. Shuto,[1] M. Nakao,[2] H. Kai[1]; [1]Molecular Medicine, Kumamoto University, Kumamoto, Japan, [2]Regeneration Medicine, Kumamoto University, Kumamoto, Japan

Myeloid elf-1-like factor (MEF) or ELF4 is an ETS protein, which has a wide distribution in hematopoietic and non-hematopoietic tissues. MEF is known to activate the promoters of granulocyte macrophage colony-stimulating factor and interleukin-3 and the transcription of perforin in NK cells. It also up-regulates the basal expression of the anti-microbial peptides, lysozyme and human beta defensin-2. We also previously demonstrated that MEF is a candidate tumor suppressor gene in the X chromosome. The numerous target genes of MEF and its biological functions signify the importance of this Ets transcription factor. Therefore, we attempted to investigate the regulation of MEF protein. Here we show that MEF is modified by conjugation with SUMO-1 (small ubiquitin-related modifier) both in vitro and in vivo at lysine 657. This modification led to the decrease of MEF activity on its target gene, lysozyme, as proven by reporter assays and RT-PCR. These data suggest that SUMO negatively regulates MEF. Another important modification that commonly occurs in histones and transcription factors is acetylation. We demonstrate here that MEF is acetylated and the functional consequence of this modification is the enhancement of MEF protein stability. Upon treatment with TSA, an inhibitor of histone deacetylase complex, MEF protein expression was enhanced. A time-course treatment with cyclohexamide, an inhibitor of de novo protein synthesis, confirmed this result. These findings provide important information on the modifications that occur on MEF and how these modifications affect its stability and activity.

481

**PLZF is a Target of the Transcriptional Repressor CDP in the Intestinal Epithelium**

I. Fréchette, F. Boudreau; Anatomy and Cell Biology, University of Sherbrooke, Fleurimont, PQ, Canada

We recently demonstrated the role of the transcription factor CDP (*CCAAT-Displacement protein*) in colonic repression of SI (Sucrase-Isomaltase) gene during development. It is well documented that SI becomes re-expressed during the first steps of colorectal cancer. However, the role of CDP in the colonic epithelium has not been yet established. In order to gain a better comprehension of CDP intestinal function, we sought to identify additional colonic transcriptional targets of CDP. We performed a microarray analysis utilizing CDP mutant mice and identified PLZF (Promyelocytic Leukemia Zinc Finger) as being upregulated in the colon of these mutant mice. Since PLZF is a transcriptional repressor implicated in the maintenance of stem cells in other systems, we undertook to verify whether CDP could modulate PLZF gene expression. First, we correlated expression of CDP and PLZF in human colorectal cancer cell lines. A Western blot analysis showed weak expression of CDP in cancer cells as compared to normal colonocytes. In contrast, PLZF was expressed in the majority of cancer cells but remained undetectable in normal colonocytes. A computer analysis of the PLZF gene predicted 21 potential interaction sites for CDP. We chose to investigate a 3 kb region upstream to the translation initiation site. A gel shift experiment confirmed the presence of five CDP-interacting sites within this region. Cotransfections of 293T cells with a CDP expressing vector and a PLZF-luciferase reporter plasmid resulted in the reduction of PLZF transcriptional activity. Finally, overexpression of CDP in the human colorectal cancer Caco2 cell line resulted in reduction of PLZF expression. Overall, these results suggest that PLZF is a direct target of CDP in the intestinal epithelium. Further studies will be necessary to unravel the biological significance of this genetic interaction.

482

**Zebrafish Contain Two Genes and Two mRNAs for AP endonuclease 1**

Y. Wang, L. F. Melo, P. R. Strauss; Biology, Northeastern University, Boston, MA

Abasic sites in DNA are produced spontaneously and by genotoxic agents. Repair is initiated by AP endonucleases, highly conserved proteins from *E. coli* to man. Since knockout of APendo is an embryonic lethal in mice, we are investigating the role of APendo in embryonic development in zebrafish *Danio rerio*. Zebrafish cDNA for APendo encodes a protein with 310 amino acids and 78% homology to mammalian APendo 1. The greatest divergence is in the amino terminus, residues 1 through 40. The expressed protein has a Km of 100 nM and a turnover number of 4 $s^{-1}$, similar to the human protein. The zebrafish genome contains two copies of a gene encoding AP endo. One copy, found on chromosome 4 (Sanger

database) consists of 4 introns ( 80-1128 bp) and 5 exons, the first of which is untranslated. Distribution of introns and exons is similar to that of the human gene. The second copy contains the same coding sequence but has no introns and lacks 700 bp found in the 3' UTR of the other copy. This copy could be a pseudogene, the result of a retrotransposon or a functional gene. Both adult and embryonic zebrafish contain two mRNAs for APendo. The larger one, which is 2.1 kb, contains the unique 700 bp found in the gene located on chromosome 4. The smaller 1.4 kb copy is missing the 700 bp found on the larger form. In short, zebrafish have two genes for APendo that are similar to the one in the human genome and two mRNAs; the encoded protein is very highly homologous to other vertebrate APendos. Supported by NIH CA72702.

483

**The Role of *Zfpm-1* on *in vitro* Erythropoiesis of Human Cord Blood CD34$^+$ Cells**

H. Yang,[1] S. Kim,[1] C. Jin,[1] K. Cho,[1] H. Park,[1] K. Chung,[1] E. Cho,[1] B. Park,[1] J. Hur,[2] T. Lee[1]; [1]Dept. of Oral Biochemistry, School of Dentistry, Chonnam National University, Gwangju, Republic of Korea, [2]Eden Hospital, Gwangju, Republic of Korea

Erythropoiesis is a complex multistage process for the differentiation of hematopoietic stem cells to mature erythrocytes. However, the molecular basis governing the functional behavior of erythropoiesis is still unclear. Previous results suggest that Prx$^{-/-}$ mice have hemolytic anemia. The EPO level in Prx II$^{-/-}$ mice was twice that of the Prx II$^{+/+}$ mice. Moreover, the number of proerythroblasts was higher in the bone marrow of Prx$^{-/-}$ mice than in Prx II$^{+/+}$ mice. There was a slightly reduced number of Lin$^-$c-kit$^+$Sca-1$^+$ cells, but there was a higher number of TER-119$^+$ cells in the Prx II$^{-/-}$ mice. This study examined the differential expression profiles using a cDNA microarray technique with bone marrow cells in Prx$^{+/+}$ and Prx$^{-/-}$ mice to determine the regulatory factors in the erythropoiesis of CD34$^+$ hematopoietic stem cells. 136 genes including 66 EST genes were differentially expressed in bone marrow cells of the Prx$^{-/-}$ and the Prx$^{+/+}$ mice. In this present, we pointed to 6 genes of RNA & protein binding group in 70 genes except 66 EST genes among differential expressed 136 genes. In order to verify functions of these interested candidates to erytheopoiesis, we established human CD34$^+$ cell culture system by using human umbilical cord blood. At day 7 of *in vitro* erythropoiesis, 4 genes, *eIF3-p44, hnRNPH1, G3bp, and Zfpm-1* were initially up-regulated and then dramatically down-regulated. However, 2 genes, *DJ-1* and *Rbm3* weren't. *Zfpm-1* siRNA treated cells contained 20% more GPA$^+$ cells than unrelated siRNA treated cells, and showed repressed expression of the hematopoietic transcription factors, *c-myc* and *c-myb*, but not *GATA-1*. These results suggest that the repression of *Zfpm-1* accelerates erythropoiesis on *in vitro* culture system. This work was supported by the Korea Research Foundation Grant funded by Korean Government (R08-2003-000-10271-0).

484

**The MEK/ERK Pathway Regulates Osteoclast Survival Through Egr1 and Egr2**

E. W. Bradley, M. J. Oursler; Endocrine Research Unit, Mayo Clinic, Rochester, MN

Reducing osteoclast numbers through targeting survival pathways may provide future therapeutic targets to slow pathological bone loss. We have shown that chemical inhibition of MEK increases osteoclast apoptosis. We therefore sought to identify downstream targets of MEK-mediated osteoclast survival. Using microarray technology, we identified two genes, Egr1 and Egr2, functioning downstream of MEK in osteoclasts. Egr1 and Egr2 expression was inhibited by chemical inhibition of MEK1/2. Further, over-expression of constitutively active MEK1 increased expression of Egr1 and Egr2. These data confirmed that Egr1 and Egr2 are downstream targets of the MEK/ERK pathway. Over-expression of Nab2, the co-repressor for Egr1 and Egr2, increased osteoclast apoptosis. Since Nab2 blocks both Egr1 and Egr2, we determined the roles of these early response genes in osteoclast survival. Over-expression of Egr1 did not repress osteoclast apoptosis, leading to the conclusion that Egr2 is the downstream effector of MEK/ERK in promotion of osteoclast survival. To determine whether Egr1 could replace Egr2 in supporting osteoclast survival, we chemically blocked MEK1/2 and determined the effect of Egr1 over-expression. Over-expression of Egr1 bypassed the effects of MEK 1/2 inhibition and decreased osteoclast apoptosis. Thus Egr1 could replace Egr2 in promoting osteoclast survival. These data demonstrate that, although Egr1 is not the downstream effector of osteoclast survival, it can substitute for Egr2 when expression is blocked by inhibiting MEK signaling. To determine whether additional targets were important in MEK-mediated osteoclast survival, we compared impacts of MEK inhibition, Nab2 over-expression to inhibit Egr family members, and combined MEK and Nab2 over-expression on osteoclast survival. When compared to chemical inhibition of MEK1/2 alone, over-expression of Nab2 in combination with MEK1/2 inhibition did not further increase osteoclast apoptosis. These data support that MEK-mediated osteoclast survival is through increased expression of Egr family members.

485

**Dynamic Behavior of Episomal DNA within the Nucleus**

J. Z. Gasiorowski, D. A. Dean; Pulmonary and Critical Care Medicine, Northwestern University, Chicago, IL

The nucleus controls many functions and interactions that are only vaguely understood at this time. Our aim is to study and characterize the mechanisms and interactions that influence episomal transgene expression within the nucleus. Contrary to widespread belief, our studies show that episomal DNA is a dynamic molecule within the nucleus. We found that plasmid DNA microinjected directly into a nucleus moves into a speckled pattern and occupies less nuclear volume than BSA, dextran, or other inert injected molecules after 4 hours. In addition, a plasmid that contains eukaryotic regulatory sequences and actively transcribes a transgene condenses into a few select areas of the nucleoplasm and occupies less nuclear volume than a bacterial vector. This suggests that episomal DNA moves in a sequence dependent manner by some means other than diffusion. We have also found that plasmids microinjected into nuclei traffic to specific subnuclear domains depending on their sequence. Our experiments show that plasmids with polymerase II regulatory elements will target to nuclear spliceosome regions, while plasmids with the polymerase I promoter often traffic into nucleoli. Alteration of episomal sequences can potentially be used to target transgenes not just into the nucleus, but also to exact regions of the nucleoplasm where expression can be maximized. Finally, we have found that directed intranclear movement of episomes into specific subnuclear domains correlates to changes transgene expression. Further elucidation of intranuclear plasmid trafficking behavior may lead to a better understanding of transgene expression that could improve basic laboratory techniques as well as clinic therapies.

486

**Ligand Activation of the Androgen Receptor Downregulates the Expression of E-cadherin in Breast Cancer Cells**

Y. N. Liu,[1] H. Z. Lee,[2] J. H. Chen[3]; [1]Graduate Institute of Medical Sciences, Tzu chi University, Hualien, Taiwan Republic of China, [2]Department

of Life Science and Institute of Biotechnology, Nation Dong Hwa University, Hualien, Taiwan Republic of China, [3]Graduate Institute of Molecular and Cellular Biology, Tzu chi University, Hualien, Taiwan Republic of China

Metastasis is a multistep process through which primary cancer cells spread to the secondary site in distant organs. To understand the mechanisms of metastasis we focused on the studies of the regulation of E-cadherin gene expression. Previously we found HNF3α could enhance human E-cadherin gene expression thus reducing the migration ability of metastatic breast carcinoma cell lines MDA435 and MDA231. In that study we noticed there was a regulatory element that exerts suppressive effect on the human E-cadherin gene expression. Gel mobility shift assay indicated this regulatory element may be a binding site for androgen receptor (AR), a member of the steroid receptor superfamily that belongs to a larger family of ligand-dependent transcription factors. Co-transfection of human full length AR cDNA with E-cadherin reporter construct showed the repression of transcription in the present of AR ligand, dihydrotestosterone (DHT). Co-transfection of HDAC1 with AR plus DHT showed the synergistic downregulatory effect on E-cadherin regulatory sequence. These data suggested AR may cooperate with HDAC1 and play suppressed effect to downregulate the E-cadherin expression in breast cancer cells. The effect of AR plus DHT on the migration ability and expression of E-cadherin on the cell membrane of MCF7 and T47D breast cancer cells will be presented.

487

**What Are Human Kao-nashi (face-less) Genes?**

N. Shimizu,[1] S. Asakawa,[1] T. Sasaki,[1] M. Furutani-Seiki,[2] H. Kondoh,[2] A. Shimizu[1]; [1]Molecular Biology, Keio University School of Medicine, Tokyo, Japan, [2]SORST, Kondoh Research Group, JST, Japan

The Human Genome Project has provided the DNA sequences of 3 billion base pairs and identified over 23,000 protein-coding genes. However, many of these protein-coding genes are not fully proven by experimental evidences. In general, proteins with known motifs are readily classified, but substantial numbers of protein have no obvious motifs in their sequences. We designated these genes/proteins without obvious motifs as KAO-NASHI (Face-less) and initiated a project to unveil their face (kao) by comparative genomics and knockdown analysis using Medaka (a small teleost fish) as animal model. We extracted 1,000 KAO-NASHI genes from 23,000 genes in the human genome using motif analysis by InterPro, homology search by BLAST and document search by PubMed. In order to unveil the functions of these KAO-NASHI genes, we decided to use Medaka (Oryzias latipes) because it is easy to obtain embryos and to observe subtle changes in the organs or anatomical structure. We have identified Medaka orthologs for over 60% of human KAO-NASHI genes using the Medaka EST and whole genome shotgun sequence databases. Developing stage of Medaka embryo was divided into 39 stages based on the diagnostic features of the developing embryos. We extracted mRNA separately from each stage and used for expression analysis of KAO-NASHI genes. Many of the initial 100 genes showed ubiquitous expression during development, but several genes showed stage-specific expression. These expression profiles are invaluable for initial functional annotation. Furthermore, we have applied the gene knockdown strategy using Morpholino oligonucleotides (MOs) injection to obtain information on the effects of a drastic reduction in expression of the target gene. In fact, we have observed some morphological changes during Medaka development after knockdown of several KAO-NASHI genes.

488

**Transgenic pS2/TFF1 Promoter-Rats: An Animal Model to Study in vivo transcriptional Mechanisms and Gene Regulation**

E. Zhong, H. Bohnemeier, M. Paul, G. Schoenfelder; Inst. of Clinical Pharmacology and Toxicology, Charité-Universitaetsmedizin Berlin, Berlin, Germany

Objective: The human pS2/TFF1 belongs to the family of trefoil peptides. TFF1 locates mainly in the cytoplasm of epithelial cells. The function of the TFF-peptides is not well understood. It was shown that they play a major role in maintaining the surface integrity epithelial cells. pS2/ TFF1 is involved in mucin polymerization, cell motility, cell proliferation and differentiation. TFF1 expression can highly be stimulated by estrogens. Several transcription factors, regulatory proteins and epigenetic factors regulate the cell-specific expression of the TFF1 gene. But, function and transcriptional mechanisms for TFF1 regulation in the female reproductive system remain unclear. Therefore, we established a transgenic rat with a TFF1 promoter-luciferase-construct to elucidate function and regulation of TFF1 under the in vivo situation. Methods and Results: Reporter assays using a TFF1 promoter-reporter, again, revealed dose-dependent response in MCF-7 cells with a series of 17beta-estradiol concentrations. After in vitro tests, we established a transgenic homozygous rat strain on a Sprague-Dawley background. PCR analysis showed that the construct was successfully integrated in the rat genome. Luciferase activity and real-time in vivo imaging of the reporter gene revealed highest transcriptional activity of the pS2-promoter in brain, uterus and vagina. Our realtime-RT-PCR analysis and immunohistochemistry confirmed transcriptional regulation of the pS2/TFF1-promoter. Conclusion: We successfully established the first TFF1 promoter-luciferase transgenic rat model to study function, regulation and morphological effects of TFF1 in a living animal model. With that animal model we are now able to study the impact of estrogens on the transcriptional regulation of TFF1 in different organs especially under in vivo conditions.

489

***Saccharomyces cerevisiae* Cells in Stationary-phase Cultures Respond to Oxidative Stress Through a Rapid Release of Sequestered mRNA**

A. D. Aragon,[1] G. A. Quinones,[1] E. V. Thomas,[2] S. Roy,[3] M. Werner-Washburne[1]; [1]Biology, University of New Mexico, Albuquerque, NM, [2]Sandia National Laboratories, Albuquerque, NM, [3]Computer Science, University of New Mexico, Albuquerque, NM

*Saccharomyces cerevisiae* cells, growing in rich medium, cease cell division and enter into stationary-phase when carbon sources are exhausted from the medium, typically after 7 days. Stationary phase is extremely important in health and disease, thus, understanding how cells survive and respond in this phase is vital. The objective of this study was to assess the ability of cells in stationary-phase cultures to respond, at the level of mRNA abundance, to oxidative stress. In response to oxidative stress (50 μM menadione final concentration) without addition of a carbon source, 1100 mRNAs exhibited significant increases within 30 minutes. To determine how quickly these transcripts appeared, a newly developed automated sampling device was used to harvest two additional time courses, with one-minute and ten-second intervals, respectively. Analysis of these time courses reveled that the increase in transcript abundance occurred within 80 seconds. There was an overlap of approximately 800 transcripts exhibiting increased abundance across all three time courses. To determine if this response was due to new transcription, an RNA polymerase II mutant, *rpb1-1,* was tested under non-permissive conditions. A similar increase in transcript abundance was observed, indicating that new transcription was not required. To test whether transcripts were in solution but not polyadenylated, mRNA was analyzed by qRT-PCR and

results indicated the transcripts were not present in isolated total RNA. Finally, cell lysates were incubated with and without several proteases, and microarray analysis showed a dramatic increase in soluble mRNA in treated samples from cells in stationary-phase cultures but not exponential cultures. Results indicate that cells in stationary-phase cultures contain a large number of intact, mature mRNAs that appear to be sequestered in protein-mRNA complexes that can be released rapidly in response to stress.

490

**DNA and mRNA Regulation of Ferritin-L is Coordinated by Hemin**

K. J. Hintze, E. C. Theil; Center for BioIron, Children's Hospital of Oakland Research Institute, Oakland, CA

Cells depend on Fe and for key reactions, in spite of potential catalysis of reactive oxygen species. Therefore, cellular Fe balance is tightly regulated; crucial to this regulation is ferritin, a highly conserved, protein nanocage that concentrates up to 4000 Fe atoms/ferritin protein overcoming the $10^{14}$-fold gradient between Fe solubility and cellular needs. In animals, ferritin is a 24mer, assembled from two gene products, ferritin-H (ubiquitous throughout nature) and L (FTL, specific to animals). Fe-overload increases FTL transcription and Fe and $O_2$ are known to increase translation. Since interactions between transcription and translation regulation have had little systematic study, we cloned the FTL promoter 1400 bp upstream of the transcription start site (TSS), where a consensus, antioxidant response/Maf recognition element (ARE/MARE; 5'-TGACTCAGC-3') occurs at -1345 bp, into a luciferase reporter construct (FTL-LUC). Treatment of FTL-LUC transfected HepG2 cells with ARE/MARE inducers sulforaphane (2μM) and tert-Butylhydroquinone (TBHQ, 10 μM), or hemin induced FTL, thioredoxin reductase or quinone reductase (ARE/MARE containing promoters) similarly; protoporphyrin IX (80 μM) also induced FTL, comparably to hemin; FAC (ferric ammonium citrate) was a relatively poor regulator. ARE/MARE mutation eliminated responses to all inducers. To explore relationships between transcriptional and translational regulation, a chimeric reporter controlled by the FTL promoter plus the cognate mRNA iron responsive element (IRE) was constructed (FTL-LUC+IRE). FAC treatment increased FTL-LUC+IRE activity 2-3-fold, while hemin treatment increased activity 10-fold. ARE/MARE mutation decreased hemin induction to the level of FAC (~2-3-fold). These results demonstrate distinct responses of the ARE/MARE to oxidative stress and the IRE to iron stress and a synergistic induction by hemin which overlaps both regulation systems possibly through the known binding to repressor proteins Bach1 (ARE/MARE) and IRP1 or IRP2 (IRE). Support: NIDDK -20251.

491

**Small Fragment Homologous Replacement Modification (SFHR) of Genomic HPRT Occurs in the Presence of p53 in Lymphoblastoid Cells**

B. Bedayat,[1] A. Abdolmohammadi,[1,2] R. Maurisse,[1] J. Schwarz,[1] J. Cheung,[1] D. De Semir,[1] J. Patrick O'Neill,[3] J. A. Nicklas,[3] D. C. Gruenert[1,2,3]; [1]Research, California Pacific Medical Center, San Francisco, CA, [2]Department of Laboratory Medicine, University of California San Francisco, San Francisco, CA, [3]Department of Medicine, University of Vermont, Burlington, VT

Gene therapy has opened the door to new therapeutic interventions for the treatment of genetic diseases. In addition to the standard cDNA-based approaches, alternative gene targeting strategies have been developed. In this study, one of these gene targeting strategies, small fragment homologous replacement (SFHR), has been used to correct a point mutation in exon 3 of the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene . This point mutation inactivates HPRT gene and allows the cells to grow in 6-thioguanine (6TG) containing medium, but not in hypoxanthine/aminopterin/thymidine (HAT) containing medium. The lymphoblastoid cell line LT1-1B1 (p53+) was transfected with a 579-bp small DNA fragment (SDF) using the AMAXA Nucleofection/Electroporation system. Several different cell to SDF concentrations were examined. The efficiency of SFHR-mediated modification was assessed in p53+ cells to determine if the presence of p53 inhibited SFHR. Corrected cells were selected in HAT containing medium and further analyzed by PCR of the DNA and by reverse transcriptase PCR (RT-PCR) analysis of mRNA-derived cDNA. Quantitative analysis showed an SFHR correction frequency of up to $10^{-2}$.

492

**A Genome-wide Search for Candidate Genes Silenced by DNA Methylation in DNMT- Knockout HCT116 Cell Lines**

C. Sers,[1] R. Kuner,[2] A. Buness,[2] P. Lund,[1] M. Ruschhaupt,[2] B. Georffy,[1] A. Poustka,[2] H. Sueltmann,[2] R. Schaefer[1]; [1]Institute of Pathology, Charité, Berlin, Germany, [2]Molecular Genome Analysis, German Cancer Research Center, Heidelberg, Germany

The covalent modification of DNA by methylation is one of the best studied epigenetic mechanisms and results in silencing of gene expression. The overall genomic methylation is lower in cancer cells compared to normal cells. However, cancer cells exhibit hypermethylation of CpG-rich regions within the regulatory regions of distinct genes. DNA methyltransferases (DNMT´s) are considered to be responsible for the maintenance of DNA methylation patterns and for the de novo methylation during embryonic development and probably tumor progression. Genes which are concerned by changes in the global DNA methylation pattern could be identified by changes in the levels of transcribed RNA. In this study, we performed a genome-wide expression study of DNMT cellular models using cDNA-microarrays (37.5K). We compared the wildtype and 5-Aza-CdR treated KRAS-transformed colon carcinoma cell line HCT-116 versus specific knockouts of DNMT1, DNMT3B and double knockout (DKO) DNMT1/3B. We found 184 candidate genes upregulated in the DNMT1 and DNMT3B single knockouts. The strongest changes in gene expression (500 upregulated genes) were observed in the DNMT1/3B DKO. The statistical analysis of Gene Ontology classes resulted in biological processes like immune response and cell proliferation which are potentially inhibited in the cancer cell line by methylation and reactivated after inhibition of both DNA methytransferases. In comparison to DNMT1/3B DKO, the gene expression profile in 5-Aza-CdR treated cells was different. Here, many genes which are involved in more general processes like transcription, translation and replication were deregulated.

493

**Identifying Heterochromatin Initiators on *D Melanogaster* Chromosome Four**

G. A. Mensah, S. C. R. Elgin, K. Haynes; Biology, Washington University in St. Louis, St. Louis, MO

Heterochromatin is vital to the organization and regulation of a significant portion of the genome; thus understanding the mechanisms involved is essential to epigentics. In contrast to euchromatin, heterochromatin is more densely packaged, is enriched in repetitious sequences, and is located around the centromeres and at telomeres. Active genes that are normally found in euchromatin become silenced once they are translocated into or near heterochromatin, due to position effect variegation (PEV). Our investigation focuses on discovering which sequences trigger heterochromatin

formation. The distal portion of *Drosophila melanogaster* chromosome four (~1200 kb) contains interspersed euchromatic and heterochromatic domains. Previous mapping studies utilizing a mobile *P* construct carrying the PEV-sensitive *white* reporter gene indicate that fragments of repetitive *element 1360* are initiators of heterochromatin in a 200 kb region of the fourth chromosome (Sun et al., 2004). We believe there are additional heterochromatin initiators; not all heterochromatic domains of chromosome four contain *1360* elements. We carried out a *P* mobilization screen to identify other heterochromatin initiators starting with a *P* element in a euchromatic domain on the fourth, where flanking variegating domains lack the *1360* element. Once mobilized, we screened for cases where the *white* reporter shows a variegated eye phenotype, presumably due to a transposition, local genomic deletion or duplication that positioned it near heterochromatin. *P* duplications can also cause silencing of *white* (Dorer and Henikoff, 1994). We are using restriction mapping to identify lines with a single intact *P* element. Subsequently, we will use an inverse PCR (iPCR) based end-mapping technique to create a genetic map pinpointing novel nucleation sites for heterochromatin formation. Of 93 variegating lines recovered, approximately 42% have a duplicated *P*, indicating that this is a major mechanism for triggering heterochromatin formation. [Supported by NIH Grant GM068388]

494

### Silkworm EST Database and Its Application to Functional Studies

K. Mita,[1] M. Morimyo,[2] K. Okano,[3] Y. Koike,[4] J. Nohata,[1] K. Yamamoto,[1] S. Sasanuma,[1] M. Sasanuma,[1] H. Kawasaki,[5] M. G. Suzuki,[6] T. Shimada,[4] M. R. Goldsmith,[7] S. Maeda[6]; [1]Genome Research Group, National Institute of Agrobiological Sciences, Tsukuba, Japan, [2]National Institute of Radiological Sciences, Chiba, Japan, [3]Microbiology, Oregon State University, Corvallis, OR, [4]Agriculture and Environmental Biology, University of Tokyo, Tokyo, Japan, [5]Agriculture, Utsunomiya University, Utsunomiya, Japan, [6]Molecular Entomology, The Institute of Physical and Chemical Research (RIKEN), Wako, Japan, [7]Biological Sciences, University of Rhode Island, Kingstone, RI

We have sequenced more than 128,000 cDNA clones from 59 cDNA libraries that were constructed from various tissues and different developmental stages to date. All ESTs have been classified into more than 17,500 groups. The comparison with FlyBase suggests that the present EST database covers more than 80% of all the genes of *Bombyx mori*. About 35,000 EST among 128,000 ESTs are compiled into "KAIKObase" accessible at the website http://sgp.dna.affrc.go.jp. [EST microarray]: We synthesized 6,000 specific primers located about 500 bp downstream from the 5'end of each cDNA to remove repetitive sequences from DNAs to be used for the microarray. 6,000 DNAs were amplified by PCR with a T3 vector primer and a specific primer, followed by spotting on glass slides. [Lepidoptera-specific genes]: Comparative studies on gene sequences between Lepidoptera, *Drosophila*, *C. elegans* and other tissues for which genome analysis is well advanced can point to Lepidoptera-specific genes. Direct links of *KAIKObase* to *FlyBase* and *WormBase* provide ready identification of candidate Lepidoptera-specific genes. The genes coding for a chitinase, a sucrase, a glucose-1-phosphatase and a glycerophosphodiesterase are supposed to be candidates of horizontal gene transfer from bacteria to Lepidoptera.

495

### Age- and Cell Cycle-dependent Changes in EPC-1 Promoter Activity in Human Diploid Fibroblast-like Cells

K. Nakahama,[1] T. Kojima,[1,2] K. Yamamoto,[3] H. Uematsu,[2] I. Morita[1]; [1]Cellular Physiological Chemistry, Tokyo Medical and Dental University, Tokyo, Japan, [2]Department of Gerodontology, Tokyo Medical and Dental University, Tokyo, Japan, [3]Department of Biology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

OBJECTIVE; The changes in gene expression during senescence are very interesting. Early population doubling cDNA-1 (EPC-1) is one of the genes whose expression decreases dramatically during cellular aging. We examined the mechanism of EPC-1 expression during cellular ageing using human diploid lung fibroblast cells (TIG-3) in culture. METHODS; TIG-3 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum. The expression levels of EPC-1 mRNA and protein were measured by real-time PCR and western blotting respectively. The EPC-1 promoter activities were determined by the Dual-Luciferase Reporter Assay System. RESULTS; The EPC-1 mRNA level of TIG-3 cells at early population doubling level (PDL) was about 3-fold higher than that at late PDL. The change in the amount of EPC-1 protein secreted into the culture medium was similar to that observed for mRNA. In TIG-3 cells at late PDL, the EPC-1 promoter activity was not changed significantly by its promoter length. In contrast, TIG-3 cells at early PDL showed a marked increase in EPC-1 promoter activity when the length of the promoter was longer than 3200 bp. Furthermore, the promoter activity was dramatically decreased after serum stimulation in TIG cells at early PDL. Conclusions; We found the promoter/enhancer region of EPC-1 existed at more than 1761bp upstream from the transcriptional initiation site of the gene, and was regulated by both aging and cell cycle. These findings suggest that the age- and cell cycle-dependent expression of the EPC-1 gene is, at least in part, regulated transcriptionally. The further analysis of the promoter region of the EPC-1 gene will reveal the mechanism(s) of cellular ageing.

## Chromatin Remodeling (496-502)

496

### Chromatin Supraorganization and Extensibility in Mouse Hepatocytes with Aging

A. S. Moraes, M. L. S. Mello; Cell Biology, UNICAMP, Campinas, Brazil

The effects of aging on chromatin supraorganization and extensibility were studied in hepatocytes of liver imprints from 1-, 2-, and 15-week-old, and 1 through 2-year-old mice. Methods used involved topochemistry, image analysis, gravity action, and polarization microscopy. Nuclear areas and Feulgen-DNA amounts were found to increase with aging in agreement with the increase in the liver cell ploidy levels. Furthermore, young and old mice showed a slightly less packed chromatin (smaller average absorbance), but more heterogenously distributed chromatin condensation levels (higher entropy), in relation to adult mice. Con-A reactive nuclear matrix glycoproteins also increased in old mice, while they were almost absent in young mice. A lysis treatment to fixed preparations induced an increased frequency of extended chromatin fiber (ECF) formation following aging. Previous works on accessibility of chromatin to nucleases described an increase in chromatin packing states in nuclei from old mice; a decrease in gene activity was also reported. The mentioned changes following aging could be a function of defects in the heterochromatin formation due to the loss of telomeres with this process, thus explaining the higher ECF formation and chromatin unpackaging in old mice. In young mice, a less packed chromatin state could probably be correlated with a more intense gene activity. Because of that, more points of

interaction between the chromatin and nuclear matrix would appear, thus inhibiting ECF formation, in spite of the less packed chromatin. The differences in content of Con-A responsive nuclear matrix glycoproteins are assumed to be related to different degrees of gene activity with advancing development and aging. (Supported by CNPq/Brazil)

497

**Cabin1 Represses MEF2 Transcriptional Activity by Association with Methyltransferase, SUV39H1**

H. Jang, H. Youn; Seoul Nat'l Univ. College of Medicine, Seoul, Republic of Korea

Cabin1, a calcineurin binding protein, was known as a transcriptional repressor of MEF2, which can be released from MEF2 in a calcium-dependent fashion. So far, the molecular basis of repression of MEF2 by Cabin1 was identified as two distinct mechanisms. Cabin1 recruits mSin3 and its associated histone deacetylases 1 and 2; Cabin1 also competes with p300 for binding to MEF2. Besides these two mechanisms, we report that Cabin1 represses MEF2 transcriptional activity by recruiting SUV39H1. First, we found that immunoprecipitated Cabin1 complex methylated Histone H3. And among histone methyltransferases, SUV39H1 associated with Cabin1 *in vivo* and *in vitro*. The SUV39H1 binding domain of Cabin1 was found to the amino acid 501-900 region. Second, SUV39H1 repressed MEF2 transcriptional activity on the Nur77 Promoter. The amino acid 501-900 region of Cabin1, which cannot recruit mSin3 and its associated histone deacetylases, also repressed transcriptional activity. Thus, Cabin1 recruits SUV39H1 and uses its histone methyltransferase activity to repress MEF2 transcriptional activity.

498

**DLAD Activity in Differentiating Lens Fiber Cells**

A. B. De Maria,[1,2] S. Bassnett[1]; [1]Ophthalmology, Washington University, St Louis, MO, [2]Biologia Celular y Molecular, Facultad de Ciencias, Universidad de la Republica, Montevideo, Uruguay

Eye lens transparency is achieved, in part, by elimination of intracellular organelles from fiber cells located in the center of the tissue. Mitochondria, endoplasmic reticulum and Golgi disappear synchronously, leading to the formation of an organelle-free zone. At the edge of this zone, nuclear breakdown involves loss of the lamina and envelope and, ultimately, chromatin degradation. Several nuclease activities have been detected in lens extracts, but only one, DLAD (DNase II-like acid DNase), has been shown unequivocally to play an active role in chromatin degradation. To better define the role of DLAD in fiber cell DNA degradation, we examined DNA fragmentation in wild type and DLAD knockout mice lenses. To visualize the DLAD expression pattern in the lens we developed an imprinting activity assay in which nuclease diffusing from a tissue slice digests an immobilized DNA substrate. The assay indicated that DLAD activity was present throughout the fiber bulk and enriched in the superficial fibers with intact nuclei. DLAD-mediated scission is believed to generate DNA free 3'-$PO_4$ ends. To detect fragmented DNA, lens tissue was TUNEL labeled with and without alkaline phosphatase (CIAP) pretreatment. In wild type lenses, TUNEL label was detected in nuclear remnants, indicating the presence of 3'-OH ends in the degraded DNA. Significantly, the amount of TUNEL labeling was not increased with CIAP treatment, suggesting that 3'-$PO_4$ ends do not accumulate. In DLAD null mice, fiber cell nuclei were not TUNEL positive, hence, DLAD may be the only nuclease responsible for chromatin degradation during nuclear breakdown. The absence of detectable 3'-$PO_4$ termini may be explained by the activity of an endogenous phosphatase that rapidly eliminates the $PO_4$ groups.

499

**Global Role of the Swi/Snf Chromatin Remodeling Complex in the Transcriptional Regulation of Glucocorticoid Receptor-Dependent Genes**

S. John,[1] T. A. Johnson,[1] C. Elbi,[1] D. A. Walker,[1] P. Meltzer,[2] A. Imbalzano,[3] G. L. Hager[1]; [1]Lab of Receptor Biol and Gene Expression, Natl Cancer Inst, Bethesda, MD, [2]Cancer Genetics Branch, NHGRI, Bethesda, MD, [3]Dept of Cell Biol, Univ of Massachusetts Med Sch, Worcester, MA

Brahma (Brm) and Brahma-related gene 1 (Brg1) serve as the ATP-dependent catalytic subunits of the swi/snf family of chromatin remodeling complexes. These complexes disrupt histone-DNA interactions and thereby provide a mechanism by which transcription factors can overcome the stearic hindrance imposed by nucleosomes. Using the well studied steroid responsive mouse mammary tumor virus promoter (MMTV), we show that both Brg1 and Brm can function on the same promoter. In Brg1 and Brm null cell lines, we find that both remodelers can potentiate the hormone-dependent response of the MMTV promoter. Immunofluorescence microscopy in cell lines with an array containing tandem repeats of the MMTV promoter show that endogenous Brg1 and Brm rapidly associate with the promoter in a dexamethasone-dependent fashion. Both biochemical and imaging approaches demonstrate that MMTV transcription is significantly reduced in cells that express catalytically inactive, dominant negative forms of Brg1 and Brm. We assessed the contribution of chromatin remodeling complexes on global gene expression by microarray analysis and found that a significant number of glucocorticoid receptor regulated genes require Brg1 for an optimal transcriptional response. This underscores a global role for Brg1 in nuclear receptor function. *In vivo* photobleaching experiments of Brg1 and Brm on the MMTV promoter demonstrated that chromatin remodeling complexes possess different kinetic properties correlating with their functional effect on MMTV transcription. Quantitative DNA fluorescence in situ hybridization experiments showed that a hormone-dependent decondensation of the MMTV array serves as an indicator of the transcriptional response and inactive Brg1 can prevent the hormone-dependent decondensation of MMTV chromatin. Thus our data indicate that MMTV transcription is regulated dynamically by multiple members of the swi/snf family of chromatin remodelers and the transcriptional response is reflected by chromatin remodeling.

500

**SWI/SNF Complex and Histone Acetylation Cooperate with STAT5 And C/EBPβ to Regulate β-casein Transcription**

R. Xu, V. A. Spencer, M. J. Bissell; Life Science Division, Lawrence Berkeley National Laboratory, Berkeley,, CA

In culture, treatment with lactogenic hormones and ECM molecules induces differentiation of mammary epithelial cells (MECs), exemplified by expression of milk proteins and formation of tissue-like structures. The gene encoding the milk protein beta-casein has been used widely as a marker for functional differentiation of MECs. Expression of the beta-casein gene is regulated by both extracellular cues and transcription factors, but how these signals lead to chromatin remodeling is not understood. Using chromatin immunoprecipitation (ChIP) assays, we now show that extracellular matrix (ECM) and prolactin cooperate to induce binding of transcription factors and SWI/SNF complex, as well as histone acetylation

in the beta-casein promoter. However, enhancing histone acetylation by treatment with a histone deacetylase inhibitor was not sufficient to induce expression of the endogenous gene. Introduction of an ATPase-deficient SWI/SNF complex significantly reduced beta-casein expression, suggesting that SWI/SNF-dependent chromatin remodeling is required for transcription. Co-immunoprecipitation analyses showed that the SWI/SNF complex is associated with STAT5, C/EBP beta, and glucocorticoid receptor (GR). These observations indicate that the functional differentiation of mammary epithelial cells requires the concerted action of both transcription and chromatin remodeling factors.

501

**Translocation Mechanism of Chromatin Remodeling Revealed by Single-molecule Studies**

Y. Zhang,[1] C. L. Smith,[2] A. Saha,[3] S. W. Grill,[1] S. B. Smith,[4] B. R. Cairns,[3] C. L. Peterson,[2] C. Bustamante[4]; [1]Department of Molecular and Cell Biology, University of Carlifornia, Berkeley, CA, [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, [3]Department of Oncological Sciences, Huntsman Cancer Institute and Howard Hughes Medical Institute, University of Utah School of Medicine, Salt Lake City, UT, [4]Department of Physics, Department of Molecular and Cell Biology, Department of Chemistry, and HHMI, University of Carlifornia, Berkeley, Berkeley, CA

ATP-dependent chromatin remodeling complexes are a large family of molecular motors that facilitate protein binding to nucleosomal DNA, transfer histone octamers in *cis* or *trans*, assemble chromatin, or exchange histones. Despite their diverse functions, these remodeling complexes share highly conserved ATPase domains and are capable of altering nucleosomal DNA configuration. However, the molecular nature of this modification and the molecular mechanisms by which it is generated remain poorly understood. Here we observe the real-time activity of two remodelers, ySWI/SNF and RSC, on single, stretched nucleosomal array templates. We show that the remodelers bind to nucleosomes and translocate DNA to form large DNA loops. Both remodelers have similar kinetic profiles and translocate at an average velocity of ~ 14 bp/s and a processivity of ~ 100 bp. Although translocation has to initiate below 7 pN of DNA tension, the remodelers can continue reeling DNA against a force up to ~ 12 pN. A DNA loop so generated either dissipates suddenly or reduces its size continuously by what appears to be a reverse translocation. Our data suggest a model for how remodelers use DNA translocation to catalyze nucleosome movements and configurational transitions.

502

***Clockswitch* is an ATP-Dependent Chromatin Remodeling Enzyme Required for Normal Circadian Regulation of the *frequency* Gene in *Neurospora crassa***

W. J. Belden, J. J. Loros, J. C. Dunlap; Genetics Department, Dartmouth Medical School, Hanover, NH

Circadian rhythms in eukaryotic organisms are established by positive and negative transcriptional/translation feedback loops. The filamentous fungus, *Neurospora crassa* contains a model circadian feedback loop that is controlled by daily oscillations in transcription of the *frequency* gene. The PAS domain containing transcription factors White Collar-1 (WC-1) and White Collar-2 (WC-2) form the White Collar Complex (WCC) that positively regulates *frq* expression in a circadian and light dependent manner. Although much is known about the transcriptional regulation of core clock components in *Neurospora*, nothing is known about how nucleosome modifications and chromatin remodeling function in regulating transcription. We demonstrate through chromatin immunoprecipitations that WC-2 binds to the *frq* promoter in a rhythmic fashion whereas WC-1 is continuously bound. We do not detect oscillations in histone H3 acetylation (H3Ac) however there is a drastic reduction in the level of H3Ac upon light induction. Nuclease accessibility experiments suggest that chromatin in the *frq* promoter changes when the gene is expressed. In order to identify the chromatin remodeling enzyme that regulates clock gene expression, a systematic deletion analysis of annotated remodeling factors was performed. We identified a single gene out of 19 annotated DNA remodeling factors that affects clock function. This gene, now designated *clockswitch* (*csw-1)* affects the transcription of *frq* as well as other genes regulated by the WCC. Antibodies generated against the N-terminal variable region indicate that CSW-1 is localized to the Clock activating activating sequence in the *frq* promoter. We also detect CSW-1 dependent chromatin remodeling at the C-box promoter element using limited MNaseI digestion followed by indirect endlabeling. These data demonstrate that chromatin remodeling is necessary for circadianly regulated gene expression.

## Nuclear Matrix and Nuclear Architecture (503-512)

503

**Nuclear Membrane, Chromatin and Nucleoli: A study of Spatial Relations in the *Didinium nasutum* Macronucleus by Means of Stereoreconstruction**

B. P. Karajan,[1] O. G. Leonova,[2] Y. F. Ivlev,[3] J. L. Ivanova,[2] V. I. Popenko[2]; [1]Institute of Cytology Russian Academy of Sciences, Saint Petersburg, Russian Federation, [2]Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Moscow, Russian Federation, [3]Severtsov Institute of Ecology and Evolution Problems Russian Academy of Sciences, Moscow, Russian Federation

A positional relationship of nuclear membrane elements, chromatin and nucleoli in the somatic nucleus of *Didinium nasutum* cyst was studied by stereoreconstruction on the basis of serial ultrathin sections. Mean concentration of nuclear pores at this stage was ~50 pores per $mkm^2$ , i.e. two times lower than at the interphase. Nuclear pores were situated on the membrane non-uniformly, but we did not succeeded in finding a correlation between location of chromatin structures and nucleoli near the nuclear membrane and nuclear pores concentration. At the cyst stage macronuclear chromatin was shown to be represented by a few individual chromatin clumps and, mainly, by fibrils of variable (100-300 nm) thickness and up to 2000 nm in length. The data obtained show that the approach combining electron microscopy, ultrathin sectioning and computer modelling allows one to build up 3D models of large areas of a cell nucleus at ~50 nm resolution and, therefore, it can be used for creation the computer models of cell nucleus functioning.

504

**Recruitment of Nup37 And Nup43 to Kinetochores: Biochemical Analyses and High Resolution Real-Time Visualisation**

S. P. Drummond, T. D. Allen; Structural Cell Biology, Paterson Institute for Cancer Research, Manchester, United Kingdom

Nuclear pore complexes (NPCs) are the gateways for both active and passive bi-directional molecular transport between the nucleoplasm and

cytoplasm during interphase. These mega-dalton assemblies are composed of multiple copies of approximately 30 distinct proteins termed nucleoporins (nups). During mitosis, in higher eukaryotes, NPCs disassemble and become dispersed throughout the cell, either as a diffuse cytoplasmic pool or are targeted to discrete sub-structures. Several nucleoporins, and presumably the nups they remain bound to during mitosis, are re-distributed to kinetochores until they are recruited back to the periphery of chromatin as NPCs are re-assembled. We have used real-time high resolution confocal microscopy to track GFP-tagged nup37 and nup43 in mammalian cells and directly observe the dynamics of these proteins throughout the cell cycle. In addition immuno-gold scanning electron microscopy (SEM) revealed the exact location of these nups during mitosis with respect to previously characterized kinetochore components. In order to investigate the requirements for nup37 and nup43 localisation to kinetochores, pSUPER constructs were used to down-regulate proteins located to kinetochore boundaries and the effects of such biochemical manipulations were again assayed using SEM and real-time deconvolution confocal microscopy.

505

### *In vitro* Assembly of Nuclear Lamins

N. Foeger,[1] N. Mucke,[2] N. Wiesel,[3] Y. Gruenbaum,[3] U. Aebi,[4] H. Herrmann[1]; [1]Department of Molecular Genetics, German Cancer Research Center, Heidelberg, Germany, [2]Department of Biophysics of Macromolecules, German Cancer Research Center, Heidelberg, Germany, [3]Department of Genetics, Hebrew University, Jerusalem, Israel, [4]M.E.Mueller Institute for Structural Biology, Biocenter, Basel, Switzerland

The nuclear lamins (NL) are nucleus-specific intermediate filament proteins that together with a complex set of membrane proteins form a filamentous meshwork tightly adhering to the inner nuclear membrane. This so-called nuclear lamina provides mechanical stability and, in addition, has been implicated in the organization of interphase chromosomes. In contrast, relatively little is known about the assembly mechanism of NLs and the specific properties of NL polymers at the molecular level. Therefore, we have more systematically investigated NL assembly *in vitro*. In particular, we identified and characterized distinct intermediates in the NL filament assembly process by negative staining and glycerol spraying/rotary metal shadowing electron microscopy. Moreover, we investigated the different assembly intermediates by analytical ultracentrifugation. Using *Caenorhabditis elegans* lamin, which has been reported to assemble into 10-nm filaments under low ionic strength conditions (Karabinos *et al*, J.Mol.Biol. (2003) **325**, 241-247), we investigated the assembly kinetics of this NL protein into filaments in more detail. Contrary to present views, we found that NLs may assemble very rapidly into large filament networks. In parallel, we investigated human recombinant lamins A/C, B1 and B2 in our *in vitro* assay system with respect to different filament assembly conditions. Last but not least, the influence of lamin-interacting proteins such as LAP2 on the assembly was explored.

506

### The Ultrastructure of Cytoplasmic Bodies formed by a Mutant Promyelocytic Leukemia Protein

D. Dinsdale,[1] C. Bellodi,[1,2] T. Smith,[1] J. McWilliam,[1] K. Kindle,[3] F. Bernassola,[4] A. Cossarizza,[2] G. Melino,[1] D. Heery,[3] P. Salomoni[1]; [1]MRC Toxicology Unit, Leicester, United Kingdom, [2]Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy, [3]School of Pharmacy, University of Nottingham, Nottingham, United Kingdom, [4]IDI-IRCCS Biochemistry Laboratory, c/o Department of Experimental Medicine, University of Rome, Tor Vergata, Rome, Italy

The tumor suppressor promyelocytic leukemia (PML) protein characterizes the eponymous nuclear bodies of normal cells but mutant forms, associated with acute promyelocytic leukemia (APL), lack the p53 interaction domain and thus the allied pro-apoptotic and growth-suppressive functions. These mutants also lack the nuclear localization signal, suggesting that they localize to the cytoplasm in APL cells. Furthermore, endogenous PML cytoplasmic isoforms have been identified, although their relationship with PML mutant forms is still unclear. Anti-PML antibodies enabled the localization of endogenous nuclear and cytoplasmic PML bodies in both normal and transformed human fibroblasts but the ultrastructure of these bodies was compromised by the mild fixation conditions required to preserve antigenicity. Primary cells were infected with retroviral supernatants from pBABE-HA-Mut1 transfected packaging cells. Antigenicity for both PML and the HA tag was detected in nuclear PML bodies of infected cells and many also exhibited double-labeled cytoplasmic PML bodies. The HA-epitope proved to be much less sensitive to fixation than PML and so samples could be prepared to retain fine cellular detail, particularly the endomembranes. The resulting samples established that the cytoplasmic PML bodies were devoid of a limiting membrane and independent of mitochondria, lysosomes and the endoplasmic reticulum. This finding was unexpected, as an association with early endosomes has been reported in some murine cells, possibly reflecting a particular stage of development or the difference in species.

507

### Knockout of Cajal Body Marker Protein Coilin Results in Decreased Transcription Efficiency

H. Xu, M. D. Hebert; Biochemistry, University of Mississippi, Medical Center, Jackson, MS

The eukaryotic cell nucleus is organized into different non-membrane bound nuclear subdomains. One of these subdomains, the Cajal body (CB), contains a host of RNA transcription and processing factors including RNA polymerase II subunits, spliceosomal ribonucleoproteins, Survival of Motor Neuron protein (SMN), and fibrillarin. As such, it is likely that this structure plays an indirect role in both RNA transcription and splicing. The marker protein for the CB, coilin, is important for CB formation and composition; coilin knockout mice demonstrate viability and proliferation problems. Embryonic fibroblasts derived from coilin knockout mice do not have canonical CBs. In contrast, two types of residual CBs, each containing a partial repertoire of CB components, are found in coilin knockout cells (Tucker et al. 2001; Jady et al. 2003). To explore CB function, we further characterized the coilin knockout cells. We found that coilin knockout cells have a significantly slower growth rate compared to wild type cells with normal CBs, consistent with a role of CBs in some aspect of transcription control or RNA processing. When challenged with exogenous RNA transcription and splicing substrates, the knockout cells demonstrate notably lower transcription efficiency as shown by RT-PCR and RNase Protection Assays, with no appreciable deficiency in pre-mRNA splicing compared to wild type cells. Thus canonical CBs, organized by coilin, appear to be critical in maintaining transcription efficiency. In order to confirm the result, we are investigating the transcription level of endogenous housekeeping gene in both wild type and coilin knockout cells.

508

**Convergence of DDX1 Foci, Cleavage Bodies, Cajal Bodies and Gems During the S Phase of the Cell Cycle**

L. Li, K. Roy, X. Sun, R. Godbout; Oncology, University of Alberta, Edmonton, AB, Canada

DDX1 foci, cleavage bodies, Cajal bodies (CBs) and gems are nuclear bodies postulated to be involved in RNA transcription, splicing and processing, perhaps by serving as pre-assembly sites for factors involved in these processes. Although all four nuclear bodies are distinct entities, they have been shown to either co-localize or partially overlap with each other. To better understand the relationship between these four nuclear bodies, we have used a three-dimensional image reconstruction program to examine the spatial distribution of DDX1 foci, cleavage bodies, CBs and gems as a function of the cell cycle. Here, we report that while DDX1 foci, CBs and gems are present throughout G1, S and G2 phases of the cell cycle, cleavage bodies are predominantly found during S phase in both HeLa and GM38 normal fibroblasts. All four nuclear bodies associate with each other during S phase, with cleavage bodies co-localizing with DDX1 foci, and cleavage bodies/DDX1 foci residing adjacent to gems and CBs. While inhibitors of RNA transcription had no effect on DDX1 foci or cleavage bodies, a dramatic alteration in the structure of cleavage bodies was observed in the presence of latrunculin B, an inhibitor of actin polymerization, suggesting a role for nuclear actin or an actin-related protein in the assembly of cleavage bodies and/or their correct positioning within the nucleus.

509

**Sp2 Localizes to Stable, Sub-Nuclear Foci Associated with the Nuclear Matrix**

K. S. Moorefield,[1] T. D. Nichols,[1] C. Cathcart,[2] S. O. Simmons,[1] J. M. Horowitz[1]; [1]Molecular Biomedical Sciences, North Carolina State University, Raleigh, NC, [2]Nikon Instruments, Inc., Melville, NY

Sp/XKLF proteins comprise a large family of sequence-specific DNA-binding proteins that share a highly conserved, carboxy-terminal DNA-binding domain. Sp2 is ubiquitously expressed and carries the least conserved DNA-binding domain amongst Sp-family members. We have reported that Sp2 binds poorly to a subset of DNA sequences bound by other family members, and has little or no capacity to stimulate transcription of promoters that are potently activated by Sp1, Sp3, and Sp4. Using an array of chimeric Sp1/Sp2 proteins, we showed further that Sp2 DNA-binding activity and *trans*-activation are each negatively regulated in mammalian cells. As part of an ongoing effort to study Sp2 function and regulation we characterized its subcellular localization in comparison with other Sp-family members in fixed and live cells. We report that Sp2 is localized largely within sub-nuclear foci, distinct from promyelocytic (PML) oncogenic domains (PODs), which are associated with the nuclear matrix. Using time-lapse confocal microscopy we report that Sp2 foci appear to be stable and virtually immobile during an 18 hour time course of observation. We also report that (1) Sp3 is distributed diffusely throughout the nucleus and only a minority of Sp3 or Sp4 is associated with the nuclear matrix, and (2) the association of Sp1 with the nuclear matrix appears to be somewhat less frequent than that of Sp2. Using a panel of partial EYFP-Sp2 fusion proteins we identified a 31 amino acid portion of the Sp2 DNA-binding domain as being necessary to direct nuclear matrix association. This portion of the Sp2 DNA-binding domain is the most divergent amongst Sp-family members. We conclude from these studies that Sp2 preferentially associates with the nuclear matrix and speculate that this association may play an important role in the regulation of Sp2-mediated transcription.

510

**Nuclear Speckles and Nucleoli Targeting by $PIP_2$-PDZ Domain Interactions**

E. Mortier,[1] G. Wuytens,[1] I. Leenaerts,[1] F. Hannes,[1] M. Y. Heung,[2] G. Degeest,[1] G. David,[1] P. L. Zimmermann[1]; [1]CME, Kuleuven, Leuven, Belgium, [2]Department of Biosciences, University of Birmingham, Birmingham, United Kingdom

PDZ domains are protein-protein interaction modules that predominate in submembranous scaffolding proteins. Recently, we showed that the PDZ domains of syntenin-1 also interact with phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and that this interaction controls the recruitment of the protein to the plasma membrane. Here we evaluate the general importance of $PIP_2$-PDZ domain interactions. We report that most PDZ proteins bind weakly to $PIP_2$, but that syntenin-2, the closest homolog of syntenin-1, binds with high affinity to $PIP_2$ via its PDZ domains. Surprisingly, these domains target syntenin-2 to nuclear $PIP_2$ pools, in nuclear speckles and nucleoli. Targeting to these sites is abolished by treatments known to affect these $PIP_2$ pools. Mutational and domain swapping experiments indicate that high-affinity binding to $PIP_2$ requires both PDZ domains of syntenin-2, but that its first PDZ domain contains the nuclear $PIP_2$ targeting determinants. Depletion of syntenin-2 disrupts the nuclear speckles-$PIP_2$ pattern and affects cell survival and cell division. These findings show that $PIP_2$-PDZ domain interactions can directly contribute to subnuclear assembly processes.

511

**Testing the Role of NuMA in Structuring the Interphase Nucleus**

A. D. Silk, P. S. Maddox, D. R. Foltz, A. Desai, D. W. Cleveland; CMM, UCSD, La Jolla, CA

It is well established that the nucleus is an architecturally complex organelle, in which chromosomes are thought to inhabit defined territories and many nuclear proteins are arranged into discrete sub-compartments. While characterization of the highly organized nature of the nucleus is well underway, few data exist which address the basic question of how, or even the degree to which the nucleoplasm is structured. A good candidate for a structural protein within the nucleus is the Nuclear Mitotic Apparatus protein (NuMA). To test a possible nuclear function for NuMA, a conditional knockout approach has been taken to generate mice that contain an allele of NuMA in which the nuclear localization sequence (NLS) is removable by Cre recombinase action. Additionally cell lines have been established that express an siRNA resistant, NLS deficient NuMA variant that complement mitotic deficits caused by knockdown of the endogenous protein, and allow an investigation into NuMA's nuclear role. Further, to analyze the function of NuMA in nuclear structure, an *in vitro* assay has been developed using spinning disc confocal microscopy. This assay is based on the property of higher order chromatin to undergo reversible restructuring in response to variation in $Mg^{2+}$ concentration. Quantitative analysis of two-color images allows simultaneous spatial and temporal resolution of changes in the distribution of chromatin (marked by fluorescently labeled histones) and nuclear sub-domain proteins. Using this assay, we found that the behavior of NPM1 (nucleophosmin, a nucleolar protein) parallels that of chromatin, while ASF/SF2 (a splicing speckle component) does not. Use of this assay, combined with siRNA-mediated removal of nuclear NuMA will determine how the removal of nuclear NuMA affects interphase chromatin distribution and the chromatin-dependent organization of multiple nuclear sub-domains.

512

### The *in situ* Response of Subnuclear Domains to Mechanical Forces Applied at the Nuclear Envelope

J. Pajerowski,[1] K. N. Dahl,[2] D. E. Discher[3]; [1]Bioengineering, University of Pennsylvania, Philadelphia, PA, [2]Cell Biology, Johns Hopkins, Baltimore, MD, [3]Cellular and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA

Nuclear plasticity, DNA bending, and related mechanical descriptors are commonly applied to the nucleus and its sub-structures, but there are few if any direct measures on cells. TC7 monkey kidney epithelial cells have been transfected with GFP Lamin A and B23, markers for the nuclear lamins and nucleolus. Micropippete aspiration of intact, suspended cells was conducted. Analysis of deformation yields information about the viscoelastic properties of the intact nucleus, as well as the nucleoli. Nucleolar deformation begins to suggest how forces are transmitted through the nucleus to subnuclear compartments. The non-homogeneous nature of intranuclear material is apparent. Nucleolar tethering events can be observed, as well as the existence of chromatin domains that exhibit varying levels of connectedness. Paralleling previous work in isolated nuclei, the creep compliance of *in situ* nuclei can be fit to a power law. In contrast to isolated nuclei, in situ nuclei exhibit a crossover from an initial power law to a more shallow power law at around 10 seconds. The second power law is similar to that displayed by swollen isolated nuclei. In order to further validate the in situ micropipette assay, cells were exposed to various treatments expected to have an effect on the physical state of the chromatin. Elevated intracellular calcium levels, induced by either selective permeabilization to divalent cations using ionophore A23187, or nonselective permeabilization using digitonin, caused a measurable increase in the stiffness of the nuclei as measured by their creep compliance. This work demonstrates that the technique of micropipette aspiration can be used to probe the structure of the *in situ* nucleus, as well as sub nuclear deformation.

## Germ Cells & Fertilization (513-534)

513

### Characterization of Allurin-Mediated Sperm Chemotaxis in *Xenopus laevis*

L. Burnett, A. Holburn, A. Bieber, D. Chandler; Arizona State University, Tempe, AZ

Allurin, a *X. laevis* sperm chemoattractant, was previously identified, purified, and characterized in our laboratory; however, the mechanics of allurin-sperm interaction leading to chemotaxis remain undetermined. Here we demonstrate allurin-sperm binding and preliminary studies for isolation and characterization of the allurin receptor on *Xenopus* sperm. Allurin binding studies were performed using both allurin conjugated with a fluorescent tracking dye and a crosslinking reagent that stabilizes the allurin-receptor complex. Oregon-Green 488 conjugated allurin, used to label sperm before, during and after activation of motility created a specific staining pattern that demonstrated the location of the allurin receptors on sperm. Furthermore, photo-crosslinking of allurin to its receptor was achieved and allowed primary separation of the receptor complex from total cell proteins. Preliminary data points to a glycosylated receptor complex greater than 150 kD molecular weight. In addition, we have begun to examine the role of specific secondary messengers in allurin-mediated sperm chemotaxis. Calcium is involved in chemotaxis in both invertebrates and mammals; we therefore began investigating what role it may play in allurin-mediated chemotaxis in two ways: fluorescent labeling with Calcium Green and disruption of calcium signaling using the ionophore ionomycin. Here we also report that disruption of calcium signaling by ionomycin results in a reduction of the ability of *Xenopus* sperm to undergo chemotaxis. This study of allurin binding and calcium signaling in sperm will lead to a model of the mechanisms and signal transduction pathways involved in sperm chemotaxis. This study was supported by NSF grant IBN-0130001.

514

### SK Channel Regulation of the Fertilization $Ca^{2+}$ Signal in Sea Urchin Eggs

C. D. Thaler,[1] C. Patton,[1] J. Maylie,[2] D. Epel[1]; [1]Stanford University, Hopkins Marine Station, Pacific Grove, CA, [2]Obstetrics and Gynecology, Oregon Health & Science University, Portland, OR

Egg activation at fertilization requires an acute rise in intracellular $Ca^{2+}$. In sea urchin eggs, $Ca^{2+}$ influx through voltage gated $Ca^{2+}$ channels and release from internal stores mediated by IP3 and/or ryanodine receptors contribute to fertilization induced $Ca^{2+}$ release. Studies with isolated microsomes suggest that an apamin-sensitive small conductance $K^+$ (SK) channel, activated by submicromolar $Ca^{2+}$, is required for optimal $Ca^{2+}$ release (Biochem J. 300:673-83). SK channel activation will potentiate $Ca^{2+}$ release by increasing the electrochemical driving force for $Ca^{2+}$ (Nature 395:908-912). To examine the potential role of an SK channel during fertilization of sea urchin eggs, the effect of microinjected SK channel blockers on the fertilization $Ca^{2+}$ signal was examined. Injection of tetrabutylamonium, TBuA, a non-selective $K^+$ channel blocker reduced the duration of the $Ca^{2+}$ wave with a dose-response similar to that for intracellular block of cloned SK channels. TBuA (~10 mM) reduced the half-width of the $Ca^{2+}$ signal from 153±3 s (n=9) to 25±1 s (n=16) and decreased the peak by 11±4%. Injection of the selective SK channel blocker, apamin, similarly reduced the duration of the $Ca^{2+}$ wave in ~1/2 of the eggs injected, consistent with restricted access to the SK channel apamin binding site which is presumably located within the ER lumen. Bath application of apamin did not affect the $Ca^{2+}$ signal suggesting that the apamin response is specific for release of $Ca^{2+}$ from intracellular stores. Similarly, block of surface membrane voltage gated $Ca^{2+}$ channels suppressed the initial $Ca^{2+}$ flash but did not affect the response to TBuA injection. These data suggest that an SK channel localized to intracellular $Ca^{2+}$ stores is activated by intracellular $Ca^{2+}$ and exerts a hyperpolarizing influence on the internal stores' membrane potential to maintain a driving force for $Ca^{2+}$ release.

515

### The Role of Phosphatidic Acid in Regulation of Intracellular Calcium during Fertilization in *Xenopus*

B. J. Stith,[1] J. Stafford,[1] Y. Chang,[1] T. Kane,[2] R. Bates,[2] A. Nickle,[2] D. Petcoff[2]; [1]Biology, University of Colorado Denver, Denver, CO, [2]Biology, Metropolitan State College, Denver, CO

The current model for fertilization in *Xenopus laevis* is that sperm somehow activates Src tyrosine kinase and that this enzyme activates phospholipase Cgamma (PLCgamma). Activation of PLC is central to fertilization as it leads to increased inositol 4,5-bisphosphate (IP3). IP3 binds an intracellular receptor to release intracellular calcium and induce subsequent fertilization events. Through Western blotting and RT-PCR that show only one isoform of PLD, we suggest that sperm activate phospholipase D1b to elevate phosphatidic acid (PA), and that PA then activates Src

tyrosine kinase which activates PLC during *Xenopus* fertilization. PA increased during *Xenopus* fertilization (from ~1 to 2.8 ng per cell; peaked at ~1 min) and that prevention of this increase inhibited fertilization. Addition of PA to eggs released intracellular calcium, and this release was inhibited by an IP3 receptor blocker (PLC inhibitor U73122 also inhibited calcium release in a plasma membrane preparation; U73343 was inactive). PA also increased IP3 mass in the absence of elevated intracellular calcium. PA or sperm addition to *Xenopus* eggs activated *Xenopus* Src (as measured by a phosphospecific ELISA kit for tyrosine 418 phosphorylation from BioSource, Inc.; PS was ineffective). As six different tyrosine kinase inhibitors reduced IP3 production or calcium release by PA (~50%), PA may activate PLC in part through activation of Src. Using Western blotting with a phosphospecific PLCgamma antibody, addition of PA to eggs not only stimulated Src activity but also increased activating tyrosine phosphorylation of PLCgamma.

516

**Where Does Extracellular Calcium Act in the Bicarbonate-Signaled Activation of Mouse Sperm?**

A. E. Carlson, B. Hille, D. F. Babcock; Physiology and Biophysics, University of Washington, Seattle, WA

The maturation of mammalian sperm within the female reproductive tract begins with an early activation of rapid motility that is produced by an increased flagellar beat frequency. The bicarbonate anion is the probable physiologic activator of this increase in motility, acting directly to stimulate the atypical adenylyl cyclase of sperm and engage cAMP-dependent protein kinase to phosphorylate components of the flagellum. Previously, we found that extracellular $Ca^{2+}$ is required for bicarbonate to increase and maintain an elevated flagellar beat frequency of epididymal mouse sperm. Here we have asked where extracellular $Ca^{2+}$ acts in this bicarbonate-signaling path. Using ratiometric photometry with the $Ca^{2+}$ sensitive indicators fura-2 or FFP18, we monitored intracellular [$Ca^{2+}$] in small groups of sperm during stimulus by perfusion with 15 mM $NaHCO_3$. Bicarbonate did not increase either cytosolic or near-membrane [$Ca^{2+}$], indicating that bicarbonate action does not produce a required entry of $Ca^{2+}$. When external $Ca^{2+}$ was removed before and during treatment with bicarbonate, intracellular [$Ca^{2+}$] decreased only slowly over several tens-of-seconds. However, the actions of bicarbonate to increase cAMP content and the flagellar beat were blocked completely within a few seconds after removal of external $Ca^{2+}$, suggesting that bicarbonate does not require a critical threshold intracellular [$Ca^{2+}$]. The most likely remaining explanation is that external $Ca^{2+}$ has a required role in the entry of the bicarbonate anion into the sperm. *Support from U54-HD12629 of the SCCPR program of NICHD. A.E.C. supported in part by NRSA T32 GM07270 from NIGMS.*

517

**Hyperactivated Motility of Sperm is Regulated by pH-Sensitive $Ca^{2+}$ Channels**

B. Marquez, S. S. Suarez; Biomedical Sciences, Cornell University, Ithaca, NY

Sperm hyperactivated motility consists of high flagellar bend amplitude and asymmetrical beating that is required for sperm penetration of the oocyte zona pellucida during fertilization and is induced by an increase in flagellar $Ca^{2+}$. Our objective was to determine the role of pH in promoting $Ca^{2+}$ signaling of hyperactivated motility. The cell-permeant weak base $NH_4Cl$ (25 mM) increased the curvilinear velocity (VCL: 345.5 ± 3.6 μm/sec vs. 200.7 ± 4.4 μm/sec control) and lateral head displacement of motile bovine sperm (ALH: 14.8 ± 0.2 μm vs. 8.6 ± 0.4 μm control), indicative of hyperactivation. Fluorometric analyses of sperm loaded with 5 μM BCECF-AM or fluo 3-AM, revealed that $NH_4Cl$ evoked elevation of intracellular pH (7.04 ± 0.05 vs. 6.86 ± 0.02 control) and $Ca^{2+}$ (262.5 ± 1.5 nM vs. 43.5 ± 3.5 nM control), respectively. Using single-cell image analysis, increased pH and $Ca^{2+}$ were detected in the flagellum. When extracellular $Ca^{2+}$ was buffered below 50 nM with 10 mM BAPTA, $NH_4Cl$-induced elevation of $Ca^{2+}$ and hyperactivation (VCL 269.5 ± 3.6 μm/sec, ALH 10.4 ± 0.2 μm) were reduced whereas alkalization was unaltered, suggesting that the rise in intracellular pH precedes the influx of $Ca^{2+}$. The $Ca^{2+}$ channel blocker $Ni^{2+}$ (100 μM) also decreased hyperactivation stimulated by $NH_4Cl$. These results indicate that signaling of hyperactivated motility involves activation of pH-sensitive channels in the flagellum that are permeable to $Ca^{2+}$ (NSF MCB-0421855).

518

**Localization and Expression of Plasma Membrane $Ca^{2+}$-ATPase in Bovine Spermatozoa and Testis**

T. Brandenburger, G. Aumüller, B. Wilhelm; Department of Anatomy and Cell Biology, Philipps-University, Marburg, Germany

Mammalian spermatozoa, like other eukaryotic cells, use $Ca^{2+}$ signals to control physiological processes. Calcium entering sperm from outside or from intracellular stores must be returned to the extracellular milieu or the intracellular storage organelles. It is known that the plasma membrane $Ca^{2+}$-ATPase (PMCA) performs the major task of $Ca^{2+}$ clearance in mouse spermatozoa. Mammalian PMCAs are encoded by four separate genes leading to four different isoforms named PMCA 1 to 4. Especially PMCA 4 has been reported to be important for male fertility. In our study we examined the protein- and gene expression of PMCA-isoforms in bovine spermatozoa and bull testis. Performing RT-PCR we could show that PMCA 1, 2 and 4 are expressed in bull testis. In situ-hybridization analysis on bull testis demonstrated a different distribution pattern of PMCA isoforms in the germ cells. Our immunofluorescence- and Western-blotting experiments on bovine epididymal sperm clearly pointed out that the PMCA-isoforms are unequally distributed. Immunoreactivity of PMCA 4 was restricted to the plasma membrane of the midpiece of epididymal sperm, while PMCA 1 immunoreactivity was observed in the acrosomal region, cytoplasmic droplet and the principal piece. We have recently shown that bovine sperm PMCA is stimulated by PDC-109, a secretory protein from bovine seminal vesicles. PDC-109 is located underneath the plasma membrane of the midpiece of bovine sperm once these got in contact with seminal vesicle secretion. Further studies have to elucidate, whether or not PMCA 4 is the isoform which is stimulated by PDC-109.

519

**A Novel Approach to Identify Bovine Sperm Membrane Proteins that Interact with Receptors on the Vitelline Membrane of Bovine Oocytes**

B. J. Pate,[1] K. L. White,[1] D. Chen,[2] P. Desai,[2] K. I. Aston,[1] B. R. Sessions,[1] B. C. Weimer[2]; [1]Animal, Dairy, and Veterinary Sciences, Utah State University, Logan, UT, [2]Center for Integrated Biosystems, Utah State University, Logan, UT

At fertilization, the sperm triggers intracellular calcium oscillations, which are pivotal to oocyte activation and development. The biological significance of these changes in intracellular calcium concentration is not fully understood. A working hypothesis for the interaction between the sperm and the oocyte is that protein ligands on the inner acrosomal membrane of the sperm bind to protein receptors on the oocyte vitelline

membrane. The aim of these experiments is to find and identify the sperm protein ligands involved in bovine sperm-oocyte interactions. In situ fluorescent labeling of proteins and 2-D gel electrophoresis were used to identify specific sperm membrane proteins that bind to membrane proteins in the oocyte vitelline membrane. Zona - free bovine oocytes were incubated in PB1+ containing Alexa Fluor 555 C2-maleimide that binds to cystein residues. Acrosome - reacted sperm were similarly labeled by incubation in Sperm TALP containing CyDye DIGE Fluor Cy5 that binds lysine residues. Unbound label was washed away from the cells, and spermatozoa and oocytes were combined in Fertilization Medium. After allowing cells to bind utilizing their native receptor/ligand complexes, the couplets were transferred into medium containing a bifunctional cross-linker, one end of which binds cystein, the other binding lysine. The cross-linked complexes were then lysed and protein extracts were examined by 2-D gel electrophoresis. The lysine and cystein labels fluoresced at different wavelengths and a scan of the gel using each wavelength was superimposed onto the other and the cross-linked protein spots were identified by their yellow coloration. These spots will be picked and proteins identified by mass spectrometry. This technique will allow identification of proteins from the bovine sperm cell that interact with proteins on the oocyte vitelline membrane.

520

**Expression of the Y-located TSPY Gene in the Rat Suggests a Role in Chromatin Remodeling during Spermiogenesis**

T. Kido, Y. Lau; Department of Medicine, VA Medical Center, University of California, San Francisco, San Francisco, CA

The Y-located testis specific protein Y-encoded (TSPY/Tspy) genes encode a family of proteins conserved at a carboxyl domain, termed TSPY/SET/NAP1 domain. The human TSPY is a repetitive gene located within the critical region of the gonadoblastoma locus on the Y chromosome (GBY) and has been postulated to be the gene for this oncogenic locus. It is expressed in spermatogonia and spermatids of normal testis and ectopically in gonadoblastoma and testicular germ cell tumors. The rat Tspy, however, is a single copy gene whose product is homologous with the human TSPY only at the SET/NAP1 domain. To investigate the possible function(s) of the rat Tspy, we have studied its expression in postnatal testes using RT-PCR and immunostaining techniques. Our results show that rat Tspy expression starts in the testis around 28 days of age. Immunostaining demonstrate that Tspy is primarily located in the elongated spermatids, different from the expression patterns of human TSPY. Comparative analysis of their promoter sequences reveals that the human TSPY promoter is CpG-rich while the rat Tspy promoter is CpG-poor, but harbors a cAMP responsive element (CRE) essential for many spermatid-specific genes. Interestingly, the rat Tspy co-localizes with the displaced histone H2B in the elongated spermatids. Since the NAP1 is involved in histone accessibility/remodeling, the co-localization of rat Tspy and histone H2B suggests that they might interact in these germ cells. Indeed histone H2B could be co-precipitated with rat Tspy using the GST-pull down assay. These results suggest that the rat Tspy may play a role in spermiogenesis by interacting with histone H2B during the chromatin remodeling in elongated spermatids.

521

**Role of Type II Phosphatidylinositol 4-Kinase in the Male Germline of *Drosophila***

J. A. Burgess,[1] H. Wei,[2] J. A. Brill[1]; [1]Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada, [2]Developmental Biology, Hospital for Sick Children, Toronto, ON, Canada

The lipid phosphatidylinositol 4-phosphate (PI4P) is emerging as a key regulator of cellular trafficking through the secretory pathway. Consistent with this view, at least two mammalian PI4-kinases, PI4KIIα and PI4KIIIβ, have been definitively localized to the Golgi where they regulate Golgi-derived secretory cargo. The *Drosophila melanogaster* ortholog of PI4KIIIβ, *fwd*, is known to be essential for cytokinesis in the male germline, but is otherwise dispensable for development. We have shown that a Fwd-GFP fusion protein localizes to the Golgi, but *fwd* mutants do not exhibit a dramatic loss of PI4P on the Golgi as assessed using a YFP-PH-FAPP reporter construct. This observation suggests that, in *Drosophila*, Fwd might be partially redundant with PI4KIIα, the other Golgi targeted PI4K. *In situ* hybridization analysis revealed that both *PI4KIIα* and *fwd* are expressed in the testes. In order to determine if PI4KIIα is required for spermatogenesis, I generated transgenic flies expressing a *PI4KIIα* RNAi 'snap-back' construct specifically in the testes. Male flies carrying this transgene are sterile and exhibit a late defect in spermatogenesis. Since RNAi might not reveal the null mutant phenotype for PI4KIIα, I used transposon based mutagenesis to generate a series of deletions all starting from the 5'end of *PI4KIIα* extending to varying distances 3'. All deletions cause embryonic lethality, suggesting *PI4KIIα* is an essential gene. To circumvent lethality and investigate the role of PI4KIIα during spermatogenesis, I have constructed a rescue construct that is expressed exclusively in somatic tissue. This construct fully rescues the lethality and will be used to observe the mutant phenotype in the germline. We conclude that PI4KIIα is required for spermatogenesis and likely represents the major PI4K activity during development.

522

**Effects of Roselle and Ginger on Cisplatin-induced Reproductive Toxicity in Rats**

A. Amin, A. Hamza; Biology Department, UAE University, Al-Ain, United Arab Emirates

Cisplatin-based polychemotherapy has been associated with high cure rates for the most frequent malignant diseases seen in young men. In view of these high cure rates, increasing clinical importance is now being attached to chemotherapy-induced fertility disorders. Cisplatin is known to induce cellular damage to testis and disrupt spermatogenesis and to cause other reproductive dysfunctions in both experimental animals and human. In this report, we used an acute cisplatin-toxicity rat model (10 mg/kg b.wt.) to analyze the protective effects of *Hibiscus sabdariffa* (HS) and *Zingiber officinalis* (ZO) against cisplatin-induced reproductive toxicity. Oral administration of extracts of HS or ZO (1 g/kg/day) for 26 days, that began 21 days before single, cisplatin intraperitoneal injection, produced significant protection of reproductive function. Both extracts reduced the extent of cisplatin-induced reproductive toxicity, as evidenced by decreases in sperm abnormality and increases in sperm motility. Cisplatin-induced alterations in testis lipid peroxidation were also markedly improved by test extracts, and cisplatin-induced alterations in the testis antioxidation defense system were profoundly prevented by extracts. In groups where cisplatin was combined with either HS or ZO, antioxidation enzymes like superoxide dismutase, reduced glutathione, and catalase were significantly elevated compared to the cisplatin-treated group. The results provide further insight into the mechanisms of cisplatin-induced reproductive toxicity and confirm the antioxidant potential of both HS and ZO.

523

### *C elegans* Sperm Cells are Damaged by Hypoxic Exposure

D. L. Miller, M. B. Roth; Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA

Cellular damage resulting from oxygen deprivation, or hypoxia, can occur during decreased oxygen availability or upon reoxygenation (reperfusion injury). It is not understood why oxygen deprivation results in cellular damage, which specific oxygen concentrations cause damage or, more importantly, how such damage could be prevented. We have been investigating the cellular response to various oxygen tensions in *C. elegans*. We show that a particular cell type, sperm, is uniquely susceptible to damage when exposed to particular hypoxic oxygen tensions. Although they are in different developmental states, hermaphrodite spermatozoa and male spermatids are equally sensitive to hypoxic exposure. Damaged sperm exhibit a motility defect *in vivo,* morphological abnormalities and fail to complete spermiogenesis *in vitro.* We are utilizing the powerful developmental genetics of *C. elegans* to explore sperm cell responses to hypoxia and mechanisms to prevent damage caused by decreased oxygen availability.

524

### Coxsackievirus and Adenovirus Receptor (CAR) is Expressed in Male Germ Cells and Forms a Complex with the Differentiation Factor JAM-C in mouse Testis

M. Mirza,[1] L. Philipson,[2] R. F. Pettersson,[1] K. Sollerbrant[1]; [1]Ludwig Institute for Cancer Research, Stockholm, Sweden, [2]Department of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden

The coxsackievirus and adenovirus receptor (CAR) is a cell surface protein that belongs to the CTX subfamily within the immunoglobulin superfamily. CAR, like several of the CTX family members, is an adhesion protein and a component of tight junctions. CAR is expressed in at least two membrane-bound isoforms that differ only at the C-terminus of the intracellular tail. We here present evidence showing that CAR is expressed in male germ cells from mice, rats and humans. CAR was detected already in round spermatids in the testis and in purified, mature spermatozoa. The two isoforms of CAR occupied different subcellular sites in the acrosomal region of the spermatozoa. Interestingly, CAR was exposed on the cell surface of acrosome-reacted but not acrosome-intact cells suggesting that CAR may have a role in the fertilization process. LNX and LNX2, two scaffolding proteins that we previously identified capable of binding to CAR, was also found to be expressed in the spermatozoa. Finally, we find CAR to form a protein complex with the spermatid-differentiation factor JAM-C. Together these findings imply a function for CAR in male fertility. These results also suggest that CAR is not accessible to adenovirus-based gene therapy vectors in the spermatozoa, and we therefore believe that the risk of CAR-mediated germ-line infection is low.

525

### Identification of Aquaporins (AQPs) in the Male Reproductive Tract of Adult Rats

L. Hermo,[1] A. M. Murphy,[1] B. Dayanandan,[1] J. Cloutier,[2] C. A. Mandato[1]; [1]Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada, [2]Neurology and Neurosurgery, McGill University, Montreal, PQ, Canada

In the male reproductive tract (MRT), water balance plays an important role in sperm transport, concentration and maturation. In many biological systems, water passes through membranes slowly by way of simple diffusion, however, certain specialized membranes exhibit a much higher capacity for water permeation due to the presence of transmembrane water channels known as AQPs. To date, several of the diverse AQP family members have been identified in the adult MRT, however, considering the extensive distribution of AQPs in the kidney and the similar embryological derivation of the two organs, we hypothesize that other AQPs family members will be expressed in the MRT. In this study, we used specific RT-PCR primers corresponding to each member of the AQP family to define their expression in the MRT. The RT-PCR results confirmed the presence of AQP 7, AQP 8 and AQP 9 transcripts in the testis, AQP 1 and AQP 9 transcripts in the efferent ducts, as well as AQP 1, AQP 3 and AQP 9 in the epididymis. In addition to these aquaporins, mRNA for AQP 5 and AQP 7 were found to be expressed in the epididymis, while AQP 7 was also present in the efferent ducts. In situ hybridization (ISH) confirmed the presence of the AQP 5 mRNA in the epididymal epithelium, using DIG-labeled antisense and sense (negative control) AQP 5 specific riboprobes. The antisense probe detected AQP 5 mRNA expression in the epididymal epithelium, with a strong reaction being present in the epithelial principal cells of all regions. In summary, our data revealed the presence of two as yet undefined AQPs (AQP 5 and 7) in the epididymis and AQP 7 in the efferent ducts.

526

### Initial Characterization of the Testis Specific Serine Kinases (TSSK) Family of Proteins in Mature Mouse Sperm

J. Sosnik, M. S. Reesse, P. E. Visconti; Veterinary and animal Sciences, University of Massachusetts, Amherst, MA

Post translational modifications of proteins through phosphorylation play a role in many cellular processes such as the transduction of extracellular signals, intracellular transport, and cell cycle progression. Our group is interested in the study of signaling events that are involved in sperm differentiation and function. Recently, a novel family of ser/thr kinases (testis specific serine kinases (Tssk)) has been found postmeiotically expressed in the male germ cells. The conserved testicular expression pattern of Tssk genes as well as the importance of phosphorylation in signaling processes strongly suggests that Tssk(s) have important role(s) in germ cell differentiation and/or sperm function. In addition, mice null for one member of this family (mTssk6) has recently been described having a male infertile phenotype (Spiridonov et al., 2005). In this work, using antibodies that recognize all the members of the Tssk family as well as some specific antibodies against individual members of the family, we show through immunofluorescence and western blots the presence of several members of the Tssk family in mature mouse sperm. This constitutes a first step to advance in the understanding of the role of this kinase family. Further biochemical characterization, as well as the analysis of null mutants (KO) of Tssk(s) will be very important for the analysis of the functions of Tssk's. If the predicted importance of Tssk's in spermatogenesis and/or sperm function is confirmed, this research can ultimately help to design novel human contraceptives.

527

### Parthenogenetic and Nuclear Transfer Embryo Development of Bovine Oocytes Denuded and Centrifuged at Different Time during Maturation

G. P. Li, T. D. Bunch, K. L. White, B. R. Sessions, K. I. Aston, B. J. Pate; Animal Dairy and Veterinary Sciences, Utah State University, Logan,

UT

This study was designed to examine the effects of removal of cumulus cells and/or centrifugation of maturing bovine oocytes on maturation and subsequent parthenogenetic and nuclear transfer embryo development. At 0, 4, 8, and 15 hr after the beginning of maturation the cumulus cells were removed and some oocytes were centrifuged followed by further maturation to 24 hr. The results showed that removal of cumulus cells or centrifugation of the denuded oocytes had no effect on the maturation rates except in the group of oocytes denuded and centrifuged at the beginning of maturation in which only 50% oocytes expelled the first polar body (PB1). However, denuding and centrifugation did affect the pattern of spindle microtubule distribution and division of chromosomes. Swollen and loose spindles were observed in anaphase I and telophase I oocytes. Activated oocytes denuded at IVM 8 hr whether centrifuged or not resulted in the lowest development (3.0%). The highest parthenogenetic development to blastocyst (17.6%) was observed in the group of oocytes denuded at the onset of maturation. Centrifugation of oocytes denuded before 8 hr of IVM was detrimental to parthenogenetic development. After nuclear transfer, centrifugation of oocytes denuded at 4, 8, and 15 hr IVM resulted in numerically lower or significantly lower blastocyst development than the non-centrifuged oocytes. However, significantly higher blastocyst development occurred in centrifuged oocytes when they were denuded at the beginning of maturation. In conclusion, the removal of cumulus cells and centrifugation of denuded oocytes affect oocyte spindle patterns. Oocytes denuded and centrifuged at different times during maturation are affected differently in terms of embryo development following parthenogenetic activation or nuclear transfer.

528

### *in vitro* Maturation of Human Oocytes in Barium Alginate Membranes

M. Torre,[1] E. Albani,[2] P. Levi Setti,[2] E. Munari,[3] U. Conte,[1] S. Villani,[4] M. Faustini,[5] G. Ricevuti,[1] E. Benzoni,[3] D. Vigo[3]; [1]Dipartimento di Chimica Farmaceutica, Università di Pavia, Pavia, Italy, [2]Istituto Clinico Humanitas, Rozzano (MI), Italy, [3]Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Università degli Studi di Milano, Milano, Italy, [4]Dipartimento di Scienze Sanitarie Applicate e Psicocomportamentali, Università di Pavia, Pavia, Italy, [5]Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Università degli Studi di Milano, Milano, Italy

Aim of the present work was to evaluate the maturation yields of human oocytes co-cultured in a pseudo-follicular granulosa cells structure. Germinal vesicles (GV) and granulosa cells were recovered from gonadotropin-treated women: GV were suspended in Ménezo Upgraded B2 INRA Medium and splitted into two groups: micro drops culture (control group) and three-dimensional co-culture (3D) group. Cumulus cells were harvested and resuspended in Ham's F10 with albumin medium at the concentration of $20 \cdot 10^6$ cells/ml. A saturated solution of $BaCl_2$ was added to the granulosa cells to a concentration of 20 mM. The suspension was then extruded through a needle and dropped into a medium viscosity sodium alginate solution 0.5% in culture medium (Ham's F10 with albumin). A single germinal vesicle was injected by means of a sterile capillary in each capsule that was finally incubated in a culture well containing 500 µl of the same culture medium at 37°C and 5% $CO_2$. GV were cultured without their cumulus masses in microdrops (25 µl) of Ménézo medium. Dishes were placed into incubator at 37°C, 5% $CO_2$. After 24 and 48 hours of culture, oocytes were classified as immature (metaphase I), or mature (metaphase II). Logistic regression shows a relevant association between success in oocytes maturation and the new culture system. Maturation probability is almost three folds higher in three-dimensional co-culture after 24 hours of incubation (P=0.001). At 48 hours the probability of maturation is greater in three-dimensional co-culture than in micro-drops one (P<0.001). A significant increase in oocyte maturation yields was obtained (co-culture: 90.3%; microdrop: 52.0%, Odds Ratio 8.51; P<0.001) in 3Dl co-culture. The risk of maturation in co-cultured oocytes is eightfold with respect to a traditional maturation technique, permitting a high number of gametes available for *in vitro* fertilization programs.

529

### Molecular and Genetic Interactions of SAX-7 with the Dystrophin-Associated Proteins, Gamma-Syntrophin and KIN-4, in the Germline

K. M. Baker, K. Opperman, L. Chen; University of Minnesota, Minneapolis, MN

The L1 family of cell adhesion molecules (L1CAMs) has been implicated in neuronal development. Non-neuronal L1CAM expression and function have been identified recently. However, their roles and mechanisms are poorly understood. We have identified requirement for the *C. elegans* L1CAM homologue, SAX-7, in multiple non-neuronal tissues, including the germline. To better determine SAX-7 functions and mechanisms, we performed a directed yeast two hybrid screen for PDZ proteins that interact with the SAX-7 consensus PDZ binding motif. We identified two proteins, gamma-syntrophin and KIN-4, both of which are associated with the dystrophin-glycoprotein complex (DGC) (1, 2). Reduced levels of gamma-syntrophin or KIN-4 accomplished by RNAi in *sax-7* mutant animals but not in wild-type animals resulted in obvious germline defects. This genetic interaction suggests that the molecular interaction between SAX-7 and gamma-syntrophin and KIN-4 is functionally significant, particularly with respect to germline development. 1. Piluso et al. (2000) J. Biol. Chem. 275: 15851-15860; 2. Lumeng et al. (1999) Nat. Neurosci. 2: 611-617.

530

### Characterization of Src Family Kinase Signaling in Echinoderm Egg Activation

I. K. Townley, M. Raisch, A. Reade, K. Foltz; Molecular, Cellular and Developmental Biology, UCSB, Santa Barbara, CA

In echinoderms (sea urchin and starfish), a current model of egg activation at fertilization involves the stimulation of at least one Src family kinase (SFK) and subsequent activation of phospholipase C gamma (PLCγ). PLCγ generates inositol trisphosphate ($IP_3$) causing release of $Ca^{2+}$ from the egg ER. Inhibition of SFK or PLCγ activity blocks $Ca^{2+}$ release. The SFK activity is upstream of PLCγ, but whether there is direct or indirect interaction between the two proteins is unknown as are the identities of other SFK substrates and interacting proteins. Immunofluorescence microscopy using affinity purified antibodies revealed that the SFKs are localized to the plasma membrane before and after fertilization, albeit with distinct labeling patterns, while PLCγ is recruited to the plasma membrane after fertilization. Biochemical methods such as co-purification via fractionation of eggs, affinity chromatography, and co-immunoprecipitation indicate that the SFK and PLCγ proteins interact in a fertilization-dependent manner, consistent with the immunolocalization data. Further, additional proteins that interact with the SFK in a fertilization-dependent manner have been detected and we are identifying these proteins. Collectively, the data suggest that there is a signaling complex in eggs that exhibits dynamic composition before and after fertilization. Biochemically, this complex can be isolated in a detergent resistant membrane fraction (lipid raft). Experiments are underway to test the effects of disrupting the signaling complex during fertilization. Supported by an NSF award to

KRF.

531

**Proteomic Analysis of Signaling at Egg Activation and during the First Mitotic Cell Cycle**

M. M. Roux, K. R. Foltz; MCDB, UCSB, Santa Barbara, CA

Post-translational modifications of the maternal proteome drive cellular activity during egg activation and throughout early embryogenesis. Changes in phosphorylation of protein modules are necessary/sufficient for successful fertilization and initiation of the developmental program. The Mitogen Activated Protein Kinase (MAPK) cascade is a highly conserved signal transduction module responsible for integrating extracellular signals whose output regulates many cellular activities. In unfertilized sea urchin eggs, constituitively active MAPK results in arrest in a haploid G0 stage of the cell cycle. Oocyte specific MAPK Kinase Kinase (Mos) is the best candidate for maintainence of this arrest. Mos has been shown to be the key component of Cytostatic Factor (CSF) which is responsible for Metaphase II arrest in vertebrate oocytes. We have recently identified and cloned the purple sea urchin (*Strongylocentrotus purpuratus*) Mos and shown that it contains an extended N-terminal domain that may be responsible for this Echinoderm specific arrest. Upon fertilization, degradation of Mos inactivates MAPK allowing for re-entry into the mitotic cell cycle. Loss of MAPK activity is sufficient for successful progression into the first mitotic S-phase. A proteomics approach has been used to identify targets of the MAPK cascade involved in release from G0 and initiation of DNA synthesis. Echinoderms naturally spawn large batches of mature eggs highly synchronized in this G0 pronuclear stage, eliminating complications stemming from meiotic regulation of this same pathway. MAPK activity can be controlled in these eggs through natural fertilization or chemical inhibitors both *in vivo* and *in vitro*. Using 2D electrophoresis, phosphorylation dependent imaging and Q-Tof MS/MS, a reproducible subset of proteins in the egg have been identified as putative targets of MAPK signaling during mitotic re-entry. The roles of these protein are currently under investigation Funding: NSFGRF, NSF IBN-0415581

532

**Computer Modeling of CUB Domains in Proteases: Binding and Enzymatic Predictions**

R. T. Almaraz, G. A. Barisone, J. L. Hedrick; Animal Science, University of California, Davis, CA

The vitelline envelope glycoproteins of amphibian eggs are processed by serine active site proteases containing CUB domains. These proteolytic modifications, namely the processing of ZPC by oviductin and of ZPAX by the hatching enzyme, are essential to fertilization and development. We hypothesize CUB domains provide specific carbohydrate binding functions to theses proteases, therefore making catalysis more efficient. To test this hypothesis, we performed 3D modeling studies and carbohydrate docking calculations on oviductin. The protease subunit of oviductin was modeled using α-chymotrypsin (pdb ID: 5cha) as a template. CUB1 was modeled after Masp2 (pdb ID: 1NTO-G) and CUB2 after C1s (pdb ID: 1NZI-A). Egg envelope oligosaccharides were used in the docking calculations. A putative carbohydrate binding site was identified on CUBs with binding specificity for high mannose oligosaccharides. We expressed and purified CUB domains for binding experiments in *E. coli*. The expressed oviductin CUB domain bound to egg envelopes. These results support our hypothesis that CUB domains, by binding to oligosaccharide moieties can act as "tethers", thereby assisting the protease domain in locating and hydrolyzing its particulate glycoprotein substrate. CUB domains are a widespread protein module, are involved in many biological processes, and are present in all metazoans. We expect our oviductin model will contribute to understanding CUB structure-function relations.

533

**Confocal Scanning Microscopy of Fluorescent Lectin-labeled Ascidian Eggs**

M. M. Botkin, R. A. Koch; Biological Science, California State University, Fullerton, Fullerton, CA

The purpose of this project is to determine the presence and localization of components of the extracelluar matrix in *Ascidia ceratodes* eggs. The ascidian egg complex, surrounded by ovijelly in the oviduct and immediately upon ovulation, is composed of a six-part vitelline coat having four non-cellular layers and two cellular components bounding the central oocyte. The non-cellular layers are the central dense layer, outer fibrous layer (OFL), inner fibrous layer, and perivitelline fibrous layer (PVFL). The cellular components consist of the follicle cells on the outer surface and test cell embedded in the PVFL and adjacent to the oocyte. Single and double labeling experiments were performed. Ascidian eggs, isolated by needle dissection, were exposed to pH 6.0 artificial seawater (ASW) to remove ovijelly, hydrated in pH 8.0 ASW, fixed in 3% formaldehyde, incubated in fluorescently labeled lectins, and photographed with a confocal microscope. Succinylated-ConA, SBA, and LPA each labeled the surfaces of follicle and test cells. UEA-1 strongly labeled the follicle cells. PNA and MPA weakly labeled the follicle cells. Succinylated-WGA labeled the OFL and follicle cells. Thus, we conclude that α-mannose, α-glucose, α & β GalNAc, α & β-galactose, GalNAc (α1, 3) Gal, and sialic acid are located on follicle and test cell surfaces. High amounts of α-L-fucose and Fuc (α1, 2) Gal (β1, 4)-GlcNAc are present on the follicle cells, whereas α & β-galactose, α GalNAc, and Gal (β 1, 3) GalNAc are weakly present on the follicle cells. Finally, β-GlcNAc and (GlcNAc-β (1, 4) GlcNAc) $_{1\text{-}4}$ are present on the OFL, surface of the follicle cells, and ovijelly. Many of these glycans co-localize. Our knowledge of these carbohydrate distribution groups will help us understanding the conditions influencing sperm-egg binding and sperm penetration. (Funded by NIH R25-GM56820)

534

**Proteomics of Fish Egg Cortical Granules III (Phosphorylated Proteins)**

K. Murata,[1] Y. Xie,[2] S. Yin,[2] J. Loo,[3] J. L. Hedrick[1]; [1]Animal Science, University of California, Davis, CA, [2]Biological Chemistry, University of California, Los Angeles, CA, [3]Biological Chemistry; Chemistry and Biochemistry, University of California, Los Angeles, CA

Egg cortical granule components play essential roles in fertilization and development in all animals. When released into the perivitelline space during the cortical reaction, these components modify the egg envelope thereby regulating sperm egg interaction (polyspermy block) and the relation between the environment and developing embryo (hardening, alternation of envelope molecular permeability). As a global strategy to determining the total macromolecular composition of cortical granules, a proteomics strategy was used. Due to advantages of size and ease of handling, Chinook salmon eggs were used as the source of cortical granule material. After activation of eggs with cold water, the perivitelline space fluid was collected by microdissection of the egg envelope (chorion). The perivitelline space fluid was dialyzed against IEF buffer, the components

separated by two-dimensional polyacrylamide gel electrophoresis, and stained with SYPRO Ruby. About 285 spots were detected. The same gel was restained with Pro-Q Diamond to detect the phosphorylated proteins. About 115 spots were detected. We analyzed the phosphorylated proteins using MS methods and preliminarily identified 48/115 spots. Supported in part by USDA 2003-35203-12831.

## Cell Polarity I (535-549)

535

### ADF/Cofilin and Neuronal Polarity

K. C. Flynn,[1,2] J. R. Bamburg[1,2]; [1]Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO, [2]Program in Molecular, Cellular, and Integrative Neurosciences, Colorado State Univeristy, Fort Collins, CO

In hippocampal neurons, the establishment of neuronal polarity occurs when the axon differentiates from one of several immature neurite processes. The family of Rho GTPases play an essential role in the initial signaling events during axon formation. In addition, a local change in actin filament dynamics in one growth cone specifies axon formation. However, the actin regulatory proteins functioning downstream of the Rho GTPases have not been identified. Likely candidates are the proteins in the actin depolymerizing factor (ADF)/cofilin (AC) family, which enhance actin filament turnover by severing filaments and increasing depolymerization rates from their minus end. Hippocampal neurons express both cofilin and ADF in about a 4:1 ratio. Here we show enhanced AC activity in engorged growth cones occurred prior to axonogenesis, although not all neurites with engorged growth cones developed into axons. Active AC remained enriched in the axonal growth cone of polarized neurons. Induction of multiple axons occurred in neurons overexpressing wildtype *Xenopus* AC (XACwt), and to a lesser degree in neurons overexpressing the non-phosphorylatable but active XAC(S3A), suggesting AC phosphocycling may be important in axonogenesis. Conversely, overexpression of inactive XAC(S3E) did not significantly affect axonogenesis. Reduction in cofilin expression by adenoviral-mediated siRNA depressed axonogenesis and neurite outgrowth, both of which are rescued by coexpression of XACwt.. Growth cone size was reduced in neurons with decreased cofilin and was increased in neurons overexpressing XACwt. Taken together, these results suggest that AC activity in growth cones regulates axon specification. (Supported by NIH grants GM35126, NS40371 and NS43115).

536

### Mechanism of Polarized Protrusion Formation on Neuronal Precursors Migrating in the Developing Chicken Cerebellum

A. Sakakibara, A. F. Horwitz; Department of Cell Biology, University of Virginia, Charlottesville, VA

Cell migration *in vivo* relies on the integration of extracellular signals, which are presented in a spatio-temporal manner in a three-dimensional environment. To elucidate the mechanism by which cells undergo migration *in situ,* we developed a novel imaging system for observing the migration of neuronal precursors in the developing cerebellum. The combination of electroporation-based gene transfer with an organotypic explant culture enabled us to label rhombic lip-derived neuronal precursors with GFP and subsequently image their dynamics. With this system, we observed that the cells show an unusually long polarized protrusion. We then studied the signaling responses that lead to this high degree of polarity. First, we demonstrated a critical role for Cdc42 in the maintenance of a monopolar shape *in vivo.* Expression of dominant negative (N17) Cdc42 abrogated polarity and resulted in rounded cells. However, perturbing downstream polarity effectors of Cdc42, e.g., the aPKC/Par3/6 complex, or APC, did not disrupt the unipolar morphology, suggesting that other downstream effectors might be involved. We next investigated the PI3-kinase pathway, which is implicated in polarized gradient sensing in *Dictyostelium* and neutrophils. Surprisingly, inhibition of PI3-kinase up-regulated non-localized branches off the long protrusion but showed no major polarity defect. Overexpression of phosphatase-dead PTEN enlarged the tip of the protrusions whereas wild-type overexpression narrowed it. Taken together this suggests that the primary function of PI3-kinase in these cells is to target active protrusions to the tip of leading process, and that PTEN may control the magnitude of protrusions rather than their localization. Finally, we showed that receptor tyrosine kinases and Rac are required for generation of the protrusions. Our results show that mechanisms of cell polarization *in vivo* share some properties with *in vitro* studies but also reveal important differences.

537

### Shootin1 is Involved in Spontaneous Neuronal Polarization

M. Toriyama,[1] T. Shimada,[1] K. B. Kim,[1] M. Mitsuba,[1] E. Nomura,[1] K. Katsuta,[1] P. Roepstorff,[2] N. Inagaki[1]; [1]Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma, Japan, [2]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Most neurons form a single axon and multiple dendrites to develop polarity. However, the mechanisms by which neurons generate only one axon to break symmetry remain poorly understood. In order to identify molecules that are involved in neuronal polarization, we performed two types of screenings of the proteome in cultured rat hippocampal neurons. One is the screening of proteins enriched in axons, and 200 positive protein spots were detected by the screening of 5,164 proteins. The other is screening of proteins up-regulated during the initial step of neural polarity formation (from stage 2 to stage 3); 277 positive protein spots were detected out of 6,197 proteins. Mass spectorometric analysis identified a novel brain specific molecule shootin1 that was detected by both screenings. Expression level of shootin1 became up-regulated and highly localized axonal growth cones during neuronal polarization. After synapse formation, shootin1 was disappeared at axonal terminus. Before polarization shootin1 began fluctuating accumulations in multiple growth cones of undifferentiated neurites, coincident with competitive elongation of the neurites. The nascent axon finally wins in a competition for exclusive and continuous shootin1 accumulation. Overexpression of shootin1 led to formation of multiple axons, whereas suppression of endogenous shootin1 by siRNA delayed neuronal polarization. Accumulated shootin1 in the growth cones recruited phosphatidylinositol 3-kinase (PI3-K) activity and mPar3 (previously reported involved in cell polarization) and induced axon formation. These observations suggest a model in which competitive interactions involving shootin1 among neurites ensure the generation of a single axon which is necessary for spontaneous symmetry-breaking in neurons.

538

### The Polarity Protein PAR-3 Mediates Dendritic Spine Morphogenesis Through Regulation of the Rac GTPase

H. Zhang, I. G. Macara; Center for Cell Signaling, University of Virginia, Charlottesville, VA

PAR-3 has been implicated in cell polarity establishment in many contexts, but the key molecular functions of the protein have been unclear. Relatively little is known about PAR-3 in neuronal polarization, and no role has been reported for any PAR protein in dendritic spine formation, a process central to the establishment of the brain circuitry. Here we show that PAR-3 is essential for dendritic spine morphogenesis in primary hippocampal neurons. Knockdown of PAR-3 expression results in the formation of multiple filopodia- and lamellipodia-like dendritic protrusions, a phenotype reminiscent of neurons expressing constitutively active Rac. PAR-3 regulates spine formation by binding the Rac GEF, TIAM1, and spatially restricting it to dendritic spines. Our data show a novel role for PAR-3 in dendritic spine morphogenesis and suggest that a principal function of PAR-3 in mammalian cell polarization is the control of actin cytoskeleton remodeling through spatial restriction of Rac-GTP formation.

539

### Schwann Cell Polarity

C. Berti, L. Wrabetz, M. L. Feltri; San Raffaele Scientific Institute, DIBIT, Milano, Italy

Three protein complexes generate polarity in several cellular contexts: 1) Par3/Par6/aPKC, 2) Crb/Pals1/PATJ-MUPP1, 3)Scrib/mDlg/mLgl. They are highly conserved throughout evolution and are part of tight (TJ) and adherens junctions (AJ). Mutations in components of these complexes lead to defects in polarity. In vertebrates, they control polarity of several cell types, including epithelia, astrocytes and neuronal growth cones, where the complex is found at the leading edge. Schwann cells surround axons and form myelin in the peripheral nervous system and, like neurons, are highly polarized cells. Similar to epithelial cells they have an apical surface facing the axon and a basal surface facing the basal lamina, but in addition they must polarize along the longitudinal axis. To explore the molecular mechanisms controlling polarity in Schwann cells, we investigated the expression of polarity complexes in sciatic nerve teased fibers and myelinating explant co-cultures. In vivo, we confirmed previous results and characterized new TJ components and polarity markers, such as PARs, claudins and claudin-associated PDZ proteins, and show that they localize in distinct regions of non-compact myelin. Surprisingly, members of the same complexes in epithelial cells do not always colocalize in Schwann cells in vivo, suggesting that Schwann cells exploit polarity molecules in a unique way. Furthermore the PAR complex may be important for myelination, as specific inhibition of aPKCζ using one of its pseudosubstrates in myelinating Schwann cells-axon co-cultures showed a reduction in the ability of treated cultures to form myelin. Schwann cells specific inhibition of aPKCζ will be performed to evaluate if the effect is Schwann cells intrinsic.

540

### Unravelling the Role of Atypical PKC-Par3-Par6 Protein Complex in Neural Proliferation and Polarity

V. Broccoli,[1] V. Belloni,[1] R. Tomasoni,[1] A. Le Bivic,[2] J. Wijnholds[3]; [1]Stem Cell Research Departement, San Raffaele Scientific Institute, Milan, Italy, [2]Developmental Biology Institute of Marseille, Faculte des Sciences de Luminy, Marseille, France, [3]Netherlands Ophthalmic Research Institute, Amsterdam., The Netherlands

Cell polarity is an essential mechanism that regulates cell adhesion and migration properties and allows compartmentalization of cell domains with specific functions. In many different cell types, cellular polarization is initiated by the restricted localization of a protein complex composed of an atypical protein kinase C (aPKCz or l), Par3, Par6. Here, we show the presence of this complex in the embryonic mammalian nervous central system and its restricted localization on the apical side of the neural progenitors. This protein complex was co-localizing with cell junction structures. We propose that this protein complex is necessary for inducing and maintaining cell-junction formation during neural tube shaping. Moreover, aPKC-Par3-Par6 complex was detected in embryonic neural stem cells. We inhibited the protein complex function by using a specific inhibitor of aPKC kinase activity. This treatment causes a general loss of proliferative activity on neural stem cells. Interestingly, aPKC inactivation leads to GSK-3b hypophophorylation and consequently to a inactivation of b-catenin. Thus, accordingly, we found that aPKC is able to bind directly to GSK-3b and to regulate its phosphorylation level. These findings reveal an unpredicted role played by the aPKC-Par3-P6 in regulating neural proliferation whose molecular mechanisms in vivo should be still carefully investigated.

541

### Annexin2 is Required for Lumen Formation in MDCK Three-dimensional Cell Cultures

F. Martin-Belmonte,[1] V. Gerke,[2] K. E. Mostov[1]; [1]Anatomy, University of California, San Francisco, San Francisco, CA, [2]Center for Molecular Biology of Inflammation, Institute for Medical Biochemistry, Munster, Germany

Lumen formation is a fundamental step in the development of the structural and functional units of glandular organs, such as kidney tubules and mammary acini. The Madin-Darby canine kidney (MDCK) epithelial cell line forms acinar structures called cysts, when embedded in a three-dimensional (3D) extracellular matrix (ECM) and represents an excellent model to analyze epithelial morphogenesis in vitro. MDCK cysts are generated from a 3D cluster of unpolarized cells that does not have a central lumen. To form the central lumen, unpolarized MDCK cells first acquire apical-basal polarity and then form de novo a central lumen surrounded by an apical belt of actin cytoskeleton. In epithelial cells, the actin cytoskeleton is regulated by the Rho family of small GTPases including the prototypical members RhoA, Rac1 and Cdc42. To exert their function, the Rho GTPases must be activated in specific plasma membrane regions. Although it has been proposed that the localization of these proteins is one of the main events that regulate their activity, the mechanisms that control the localization of the Rho GTPases are still poorly understood. Here, we show that the phosphatidylinositol (4,5) bisphosphate binding protein annexin2 tethers Cdc42 to the apical membrane of epithelial MDCK during cysts formation. Furthermore, when the function of annexin2 is disrupted by the expression of the dominant negative forms of this protein, Cdc42 is mislocalized causing the disorganization of the apical actin belt and the inhibition of central lumen formation.

542

### Examination of Mammalian Par 6 Nuclear Function

E. G. Cline, W. J. Nelson; Molecular and Cellular Physiology, Stanford University, Stanford, CA

In mammalian epithelial cells, Par 6, Par3, and aPKC form a complex localized to cell membranes in the region of the tight junction. This complex is important for cell polarity. Immunofluorescence studies of MDCK II cells with an affinity-purified antibody have shown that Par 6-alpha is localized not only to the apical junction region of cells, but also in distinct "speckles" in the nucleus. This nuclear localization has been confirmed biochemically and in several different cell lines. Nuclear localization of Par 6 suggests a role in gene expression either through transcription or

mRNA maturation and transport. Fluorouridine incorporation assays and GAL-4 transactivation assays indicate that Par 6 is not involved in activating transcription. Par 6 nuclear speckles colocalize with the spliceosome component SC35 and with overexpressed Paraspeckle Protein -1, suggesting a role in post-transcriptional mRNA biogenesis. The Par 6 nuclear staining pattern also changes in response to transcription inhibiting drugs in the same manner as proteins involved in splicing. Expression of the HTLV-1 viral transcriptional transactivation protein Tax, which interacts with Par 6, causes aggregation of transcription and splicing factors, and Par6 in the nucleus. Taken together, these results indicate roles for Par6 in gene expression and mRNA biogenesis.

543

**Hepatic Progenitor Cells Establish Epithelial Polarity in the Three Dimensional Culture**

N. Tanimizu,[1] A. Miyajima,[2] K. Mostov[1]; [1]Anatomy, University of California San Francisco, San Francisco, CA, [2]Institute of Cellular and Molecular Bioscience, University of Tokyo, Tokyo, Japan

The mammalian liver contains two types of epithelial cells, hepatocytes and cholangiocytes. Both of them originate from hepatoblasts. Mouse cholangiocyte differentiation around portal vein starts at about embryonic day 15. Immature cholangiocytes make ductal plates, generate the apical lumen between two cell layers, and then form bile duct tubules. Several mutant mice and human infants have been reported to show defects in bile duct development, which cause cholestasis, fibrosis, and cirrhosis of liver, though the functions of the mutated genes in cholangiocyte morphogenesis have not been clear. One of the reasons is that there is no *in vitro* culture system applicable to study cholangiocyte differentiation from hepatic progenitor cells. We established a liver progenitor cell line, HPPL, from mouse hepatoblasts (1). Since HPPL preserves proliferative capability and multi-differentiation potential of hepatoblasts, it is useful to study the process of bile duct formation from their progenitors. We are applying the three dimensional culture system in which cells grow in a gel of extracellular matrix proteins to understand the mechanism of cholangiocyte polarization and tubulogenesis. In collagen gel, HPPL proliferate and form extended structures by the stimulation of HGF. However, these structures did not have an apical lumen. On the other hand, when HPPL was kept in the mixture of Matrigel and collagen, some of HPPL formed cysts that had the apical lumen. These cysts expressed cytokeratin 19, which is a marker of cholangiocytes, suggesting HPPL developed the epithelial polarity as cholangiocytes. Considering that cholangiocytes are associated with the basement membrane during embryonic development, the interaction between HPPL and components of Matrigel induced the polarization of HPPL in the culture. 1. Tanimizu, N., Saito, H., Mostov, K., and Miyajima, A. (2004) *J. Cell Sci.* **117**, 6425-34.

544

**Cdc42 Regulation of Atypical Protein Kinase C**

S. X. Atwood, K. E. Prehoda; Institute of Molecular Biology, University of Oregon, Eugene, OR

Atypical protein kinase C (aPKC) is a key regulator of cell polarity. aPKC forms a complex with Bazooka/Par-3 and Par-6 that functions to separate apical and basolateral plasma membrane domains of epithelia and asymmetrically localize cell fate determinants in stem cells. The small Rho GTPase Cdc42 interacts with this complex through an interaction with Par-6 that is thought to be important for regulating aPKC activity. However the mechanism by which Cdc42 alters aPKC kinase activity through Par-6 is poorly understood. We show that binding of Cdc42 does not remodel the Par-6/aPKC complex, but instead modulates a secondary interaction between the CRIB-PDZ protein interaction domain of Par-6 and aPKC. We also show that Cdc42 localizes to the cytoskeleton and not the membrane of Drosophila embryos where presumably aPKC can bind to and phosphorylate its ligands. Our data provides evidence for an allosteric model for the regulation of aPKC activity by Cdc42 as well as a novel model for the localization of the Par-6/aPKC complex to the cell periphery.

545

**Evidence for Epithelial-to-Mesenchymal Transition in a Mouse Model for Autosomal Recessive Polycystic Kidney Disease**

S. P. Ryan, C. Carlin; Dept of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

Epithelial-to-mesenchymal transition (EMT) is a highly regulated biological event with important roles during embryonic development, tissue repair, and tumor metastasis. EMT is well studied in the kidney, where epithelial cells lining nephrons are either shed into the lumen and die, or transition to fibroblasts, following renal injury. Autosomal Recessive Polycystic Kidney Disease (ARPKD), characterized by epithelial cell hyperproliferation and acquisition of a secretory phenotype leading to cyst formation, is a devastating disease affecting approximately 1:20,000 persons annually. We hypothesize that ARPKD represents a failed attempt at EMT. If true then cystic cells should exhibit a subset of characteristic EMT features. We tested this hypothesis using a cell culture system developed by mating BPK mice [bpk (+/-)], a murine model of ARPKD, with the Immorto-Mouse [H-2K(b)-ts-A58(+/+)]. Principal cell lines were isolated from the kidney collecting tubule segments from either cystic or non-cystic animals. Cells are grown in a chemically defined media on permeable filter inserts, which allows for polarization of the epithelial cells and domain-specific experiments. When looking at particular markers for EMT, we discovered that N-cadherin is overexpressed in the cystic cells. N-cadherin is a mesenchymal marker, typically induced during EMT. This overexpression was verified through both western blotting of cellular proteins and confocal microscopy. We have shown previously that the EGF receptor (EGFR) is found predominantly on the basolateral surface in normal cells, but is more evenly distributed on both apical and basolateral surfaces in cystic cells. Since the epithelial cell adhesion molecule E-cadherin is an important landmark for basolateral membrane cargoes, we hypothesize that overexpression of N-cadherin in the cystic cells is a contributing factor to EGFR mislocalization, which has been linked to hyperproliferation and cyst expansion and ARPKD.

546

**The Keratin-binding Domain of the γ-Turc Component GCP6 is Important for MTOC Localization and Architecture of Microtubules in Polarized Epithelial Cells**

A. S. Oriolo, F. A. Wald, P. J. Salas; Cell Biology and Anatomy, Univ. of Miami School of Medicine, Miami, FL

This work was intended to determine the molecular mechanisms that anchor γ-tubulin-containing structures to intermediate filaments (IFs), and to determine if apical non-centrosomal microtubule (MT) organizing centers (MTOCs) are important in the nucleation of MTs and stabilization of the *minus* ends in polarized epithelial cells. A screen of mammalian cDNA libraries by yeast-two hybrid showed that a 190 kDa component of the γ-tubulin ring complex (γ-Turc), GCP6, binds keratins. This finding was independently confirmed by expressing full-length HIS-tagged GCP6 and

using it in Far-Western analysis and pull-downs on purified keratins and IFs. Likewise, the same keratin-binding properties were shown for the C-terminal of GCP6 alone. Co-immunoprecipitation indicated that this is the same CDK1-responsive protein previously identified as a component of the MTOC/IF interphase. shRNA knock-down of GCP6 resulted in changes in the distribution of γ-tubulin, the architecture of MTs, and the localization of the MT nucleating activity in CACO-2 cells. Overexpression of the keratin-binding domain alone under the Tet-responsive promoter also resulted in redistribution of the γ-tubulin signal and basolateral nucleation of MTs. The HA-tagged GCP6 keratin-binding domain colocalized with IFs in vivo. MT nucleation experiments did not show any activity at the centrosomes, except in mitotic cells, but rather showed broadly distributed nucleation across the apical cortical region of CACO-2 cells, coincidental with the localization of non-centrosomal γ-tubulin in polarized cells. These results highlight the importance of IFs as a cortical scaffold for MTOC localization, shed light on the molecular mechanisms responsible for disorganization of MTs in K8 knock-out enterocytes, and are compatible with a model of MT nucleation at small non-centrosomal MTOCs in polarized epithelial cells.

547

**Role of the Crumbs Complex in the Morphogenesis and Homeostasis of Epithelia**

A. Le Bivic, M. Delgrossi, D. Michel, J. Arsanto, C. Beclin, D. Massey-Harroche; NMDA, IBDM, Marseille, France

Epithelial polarity is essential for the normal physiology of multicellular organisms since epithelia protect from the external environment and allow communication and exchanges with it. Control of cell polarity and proliferation in epithelial layers is also crucial for the homeostasis and shape determination preventing the development of polyps and tumors. Several protein complexes important for the development and the maintenance of epithelial cell polarity have been identified in the last years. Among these complexes, the Crumbs complex has a conserved role in the building and maintenance of epithelial apical junctions (Zonula Adherens in Drosophila and tight junctions (TJs) in mammals) acting coordinately with the PAR3/PAR6/aPKC complex. In mammalian epithelial cells, the Crumbs complex is made of Crb3, Pals1 and Patj and the exact contribution of each of these proteins in the mechanisms controlling epithelial homeostasis is not yet understood. We have used a human intestinal epithelial cell line, Caco2, as a model to understand the role of each member of the Crb3 complex by using a RNA interference strategy. We have studied in this work the impact of Patj knock down (KD) on cell polarity, TJ formation and proliferation in Caco2 cells. We have observed that several markers of TJs (Occludin and ZO-3) are mislocalized on the lateral membrane in Patj KD cells while Crb3 and Pals1 are re-distributed in intracellular compartments indicating that Patj acts as a linker between the Crb3 complex and the TJ. Moreover, we have found that Patj interact directly with the Tuberous Sclerosis complex that give multiple hamartomas when mutated in human. Patj acts as an regulator of the mTOR pathway through TSC and is thus the first component of the Crb complex involved in the control of tissue growth and homeostasis.

548

**aPKC/Par Proteins Show Asymmetric Localisation in Lens Epithelial Cells**

Y. Sugiyama,[1] A. R. Prescott,[2] R. A. Quinlan[1]; [1]School of Biological and Biomedical Sciences, Durham University, DURHAM, United Kingdom, [2]School of Life Sciences, University of Dundee, Dundee, United Kingdom

The eye lens comprises the anterior lens epithelial cells and its differentiated derivative, the lens fibre cells. Both cells have apico-basal polarity and cell polarity is expected to have important role for their physiological function and maintenance of cell shape. Since lens cell polarity is also maintained during lens development it suggests that is involved in lens formation as well. To analyse the role of cell polarity in the lens, we have tried to determine if aPKC/PAR machinery is responsible for lens cell polarity formation. aPKC/Par3/Par6 are major epithelial cell polarity proteins and the establishment of polarity is accompanied by their asymmetric localisation to the tight junction (TJ) region of epithelial cells. Using adult bovine lenses, we have found that aPKC/Par3/Par6 all locate asymmetrically at the apical cell contact region of lens epithelial cells. This region was also positive for the TJ proteins, ZO-1/occludin/claudin-1. The finding of expression of TJ specific proteins, occludin and claudin-1 in the lens was a somewhat surprising result as it has been previously reported that lens epithelial cells do not posses classical TJ structures. Furthermore, we detected the adherens junction (AJ) proteins, E-cadherin and N-cadherin, and a gap junction protein, Cx43, in the same apical cell contact region. The asymmetric localisation of aPKC/Par3/Par6 suggests this protein machinery is involved in establishing lens cell polarity. The finding that the apical cell contact region of the lens epithelial cell contains TJ, AJ and gap junction proteins simultaneously suggests lens epithelial cells develop atypical cell junction structure comparing to the typical epithelial cell junction. To clarify this particular junctional structure, we are now analysing it using immuno-electron microscopy. To indicate direct involvement of aPKC/PAR in lens development and analyse their roles, we have been developing mouse aPKC knockout lenses.

549

**Numb Controls Integrin Endocytosis for Directional Cell Migration with Par-3 and aPKC**

T. Nishimura, K. Kaibuchi; Cell Pharmacology, Nagoya University, Nagoya, Japan

Cell migration is characterized to extend a leading edge protrusion and to establish new adhesion sites at the front. Integrins are major cell adhesion receptors and play key roles in development, immune response, and cancer metastasis. Polarized membrane recycling including integrin is thought to be one of the driving forces for cell migration. However, molecular mechanisms for how integrins are internalized at the specialized cell area are largely unknown. We here found that endocytic protein Numb preferentially localized at the substratum-facing surface and polarized away toward the leading edge. Numb is a PTB domain containing adaptor protein implicated in clathrin-dependent endocytosis by binding to Eps15 and AP-2 complex. Numb bound to integrin-bs, and suppression of Numb inhibited integrin endocytosis, but not transferrin endocytosis. Correlated with the defects of integrin endocytosis, suppression of Numb impaired cell migration toward integrin substrates. Furthermore, Numb interacted with the polarity complex PAR-3 and was directly phosphorylated by aPKC. aPKC phosphorylation controlled the membrane localization of Numb, and thereby resulted in the defects of integrin endocytosis and integrin-stimulated cell migration. These observations suggest that Numb mediates integrin endocytosis at the substratum-facing surface for polarized cell migration, and that localization and functions of Numb are regulated by PAR-3 and aPKC.

## Neurotransmitters, Peptides & Receptors (550-569)

550

### Chromogranin A Deficiency in Transgenic Mice Leads to Aberrant Chromaffin Granule Biogenesis

T. Kim,[1] C. Zhang,[1] Z. Sun,[2] H. Wu,[2] Y. P. Loh[1]; [1]NICHD, NIH, Bethesda, MD, [2]Peking University, Beijing, China

Dense-core secretory granules (DCGs) store and secrete neurotransmitters, hormones and neuropeptides in neuroendocrine and endocrine cells. In adrenal chromaffin cells, DCGs store and secrete catecholamines (CA), pro-enkephalin and chromogranin A (CgA) upon the stimulation by acetylcholine. Chromogranin A (CgA) is a highly abundant soluble glycoprotein present in DCGs. CgA binds CA for storage in the lumen of chromaffin granules, and it is co-released along with CA in a regulated manner. Recently CgA was shown to be a key regulator of DCG biogenesis at the trans-Golgi network in neuroendocrine PC12 cells (Kim et al., 2001 Cell 106:499-509). To study the role of CgA in DCG biogenesis *in vivo*, we generated transgenic mice expressing CgA antisense RNAs. CgA antisense transgenic mice showed significant reduction in CgA expression to 30-70% of wild type animals in adrenal glands. In addition, other granule proteins such as chromogranin B, carboxypeptidase E and dopamine β-hydroxylase were also decreased significantly in the adrenal of these mice. The reduction of CgA led to significant depletion (~30-50%) of DCG formation in adrenal chromaffin cells from these transgenic mice. The quantity of DCGs formed in the transgenic mice was directly correlated with the amount of CgA in adrenal medulla. Interestingly DCGs formed in transgenic mice were considerably enlarged in the luminal volume around the dense core, a phenomenon that occurs to maintain constant "free" CA concentration in the lumen of these granules. DCG swelling was correlated inversely with the quantity of DCGs formed in the adrenals, thus with the quantity of CgA in these mice. Our data indicate that CgA play an important physiological role in regulating chromaffin granule biogenesis and CA storage *in vivo.*

551

### The Effect of Somatostatin on the Release of $^3$H-1-methyl-4-phenylpyridinium from Bovine Adrenal Chromaffin Cells

L. Ribeiro, F. Martel, I. Azevedo; Biochemistry, Faculty of Medicine, Oporto, Portugal

Besides cholinergic regulation, catecholamine secretion from adrenal chromaffin cells can be elicited and/or modulated by noncholinergic neurotransmitters and hormones. This study was undertaken to investigate the influence of somatostatin on [$^3$H]MPP$^+$ secretion, evoked by KCl or cholinergic agents, from bovine adrenal chromaffin cells**.**The release of [$^3$H]MPP$^+$ from these cells was markedly increased by excess (50 mM) KCl (to 542±60 % of basal release, n=23). At concentrations between 50 μM and 10 mM, acetylcholine (ACH) significantly increased the release of [$^3$H]MPP$^+$ . The effect of ACH was concentration-dependent until 1 mM, where a maximal stimulation of 483±64 % over basal release was observed. However, the highest concentration of ACH (10 mM) induced a more modest increase than the previous dose (265±24 % over basal release). The acetylcholine-evoked release of [$^3$H]MPP$^+$ from these cells was mainly mediated by nicotinic receptors since: a) nicotine and 1,1-dimethyl-4-phenylpiperazinium (DMPP), selective nicotinic agonists, stimulated the release of [$^3$H]MPP$^+$, b) a nicotinic antagonist, hexamethonium, markedly blocked the acetylcholine-evoked response and c) pilocarpine, a muscarinic agonist, was devoid of effect on [$^3$H]MPP$^+$ secretion. At all concentrations tested, somatostatin and octreotide interfered neither with [$^3$H]MPP$^+$ basal release nor with KCl-induced release of [$^3$H]MPP$^+$. On the other hand, somatostatin (0.01-0.3 μM) increased the release of [$^3$H]MPP$^+$ induced by a high concentration (10 mM) of acetylcholine (to about a maximum of 210% over ACH-induced release) but, at higher doses (1 and 10 μM) was devoid of effect. Octreotide (1-10 μM) had no effect. These results support the hypothesis that somatostatin may increase the release of catecholamines from adrenal medullary cells.

552

### Control of Catecholamine Release from the Adrenal Medulla of $\alpha_{2a}$, $\alpha_{2b}$ and $\alpha_{2c}$ KO Mice

E. Moura, J. Afonso, H. de Sousa Lopes, M. A. Vieira-Coelho; Institute of Pharmacology and Therapeutics of Porto University, Porto, Portugal

$\alpha_2$-adrenoceptors have been reported (Brede et al, 2003) to inhibit adrenaline release from cultured adrenal chromaffin cells of mice. The aim of this work was to evaluate the effect of the $\alpha_2$-adrenoceptor agonist dexmedetomidine (MED) on catecholamine release from the adrenal medulla of knock out mice for each of the three $\alpha_2$-adrenoceptor subtypes: $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$. Isolated adrenal medullae from control (CT) mice and $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$ KO mice aged 30 weeks, were placed in suprafusion chambers and continuously washed with Krebs-Henseleit solution at a rate of 0.5mL.min$^{-1}$ for 90 minutes. Samples of suprafusion fluid were then collected every 5 minutes. Dimethylphenylpiperazinium (DMPP) (500μM) was added to the fluid for 5 minutes either in the absence or in the presence of MED (100nM). The assay of catecholamines in the tissues and in the fluid was carried out by HPLC-ED. Results are given as arithmetic mean±se. Statistical analysis was done by ANOVA. DMPP increased the release of NA and AD in all strains (CT: 4.59, 2.56; $\alpha_{2A}$: 4.18, 3.13; $\alpha_{2B}$: 3.30, 2.17; $\alpha_{2C}$: 4.66, 2.35 % increase in release, NA and AD respectively, n=5). The inhibitory effect of MED on NA induced release was similar between the KO mice strains (CT: 89; $\alpha_{2A}$: 33; $\alpha_{2B}$: 38; $\alpha_{2C}$: 36 %, n=5) whereas for AD induce release it was less marked for the $\alpha_{2C}$ KO mice (CT: 90; $\alpha_{2A}$: 54; $\alpha_{2B}$: 43; $\alpha_{2C}$: 29 %, n=5). In conclusion, although all three $\alpha_2$-adrenoceptor subtypes play a role in the regulation of the spontaneous release of NA and AD from the adrenal medulla of mice, the $\alpha_{2C}$ subtype might be the predominant subtype for AD release. Brede M. et al (2003) Mol Endocrinol 17:1640-1646. Supported by grant POCTI/40832/FCB/2001.

553

### Role of Alpha 1-adrenoceptor Subtypes in the Cardiac Failure Induced Sprague Dawley and Spontaneously Hypertensive Rats

S. Atif Abbas,[1] M. Zubaid,[2] M. N. Noriah[1]; [1]Faculty of Pharmacy, International Islamic University, Kuantan, Malaysia, [2]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia

The function of kidney deteriorates during the development of cardiac failure because of changes in renal haemodynamics and neurohormonal activity. This study aimed to examine the alpha 1 adrenoceptor subtypes involved in mediating adrenergically induced renal vasoconstriction in a rat model of cardiac failure and hypertension. Normal Sprague Dawley (SD) and spontaneously hypertensive rats (SHR) were used in the study. Cardiac failure was induced by the combined treatment of caffeine (40mg/kg) and isoprenaline (5mg/kg) for seven days. On day eight, the rats were used for acute study. The left kidney was exposed, the renal artery cleared and an electromagnetic flow probe on it for renal blood flow (RBF) measurements. The reduction in renal blood flow induced by increasing frequencies of electrical renal nerve stimulation, close intrarenal bolus doses of noradrenaline, phenylephrine or methoxamine were determined before and after administration of amlodipine, 5-methylurapidil,

chloroethylclonidine and BMY 7378. Data, means ± s.e.m were compared with 2 way ANOVA followed by Bonferroni post hoc with the significance level of 5%. The results obtained indicated that the renal vasoconstrictor responses in this model were attenuated mainly by amlodipine, 5 methylurapidil and BMY7378 but not by chloroethylclonidine. Furthermore, administration of chloroethylclonidine did not show a significant reduction in methoxamine induced renal vasoconstriction in cardiac failure SD and SHR. This supported the view that alpha 1 A-adrenoceptors are involved in renal vasculature SD and SHR regardless of its pathophysiological state. The findings from this study further suggested that besides the alpha 1A the alpha 1D- adrenoceptors also contribute to the adrenergically induced renal vasoconstrictor responses in cardiac failure SD and SHR.

554

**Agonist Induced Internalization and Lipid Raft Trafficking of G alpha s Regulates Adenylyl Cyclase**

J. A. Allen,[1] J. Z. Yu,[1] A. Bhatnagar,[2] B. L. Roth,[2] M. M. Rasenick[1]; [1]Department of Physiology and Biophysics, University of Illinois at Chicago-College of Medicine, Chicago, IL, [2]Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH

Many G protein-coupled receptors are internalized by endocytosis and recent studies have indicated that some G proteins also undergo agonist induced internalization. Previously, our laboratory has shown that stimulation of Beta-adrenergic receptors (BetaAR) results in lipid raft-mediated internalization of G alpha s (Gs). The objective of this current study is to determine whether agonist induced internalization of Gs from lipid rafts regulates cAMP synthesis. C6 glioma cells or C6 cells in which caveolin-1 was stably knocked down by RNAi (C6 Cav-1) were transfected with Gs-GFP and trafficking was assessed using fluorescence microscopy. Upon stimulation of C6 cells with the BetaAR agonist isoproterenol, Gs-GFP was rapidly removed from the plasma membrane and internalized within vesicles. However, Gs-GFP internalization was blocked by disrupting lipid rafts/caveolae with cyclodextrin and other cholesterol disrupting drugs. Subcellular fractionation studies revealed that agonist treatment significantly increased Gs localization in Triton X-100 insoluble lipid raft membrane fractions, while BetaARs were removed from lipid rafts. In addition, cyclodextrin disruption of rafts significantly increased isoproterenol and forskolin stimulated adenylyl cyclase activity. In experiments studying C6 Cav-1 cells, Gs-GFP did not internalize during agonist treatment, indicating that rafts/caveolae are necessary for Gs internalization. In addition, isoproterenol and forskolin stimulated adenylyl cyclase activity was significantly increased in the C6 Cav-1 cells vs. wild type C6 cells. These results suggest that during receptor activation, Gs is internalized through lipid raft/caveolae microdomains of the plasma membrane where it is less available to adenylyl cyclase, diminishing cAMP synthesis. We suggest that lipid rafts/caveolae act as negative regulators of BetaAR/Gs/adenylyl cyclase signaling.

555

**Trafficking of AMPA Receptors at Synapses is Regulated by LIN-10 and RAB-10**

D. R. Glodowski,[1] C. Chen,[2] B. D. Grant,[2] C. Rongo[1]; [1]Waksman Institute, Rutgers University, Piscataway, NJ, [2]Dept of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ

In neurons, the regulated cycling of AMPA-type glutamate receptors (AMPARs) into and out of the synapse leads to changes in synaptic strength that are thought to be important for processes like learning and memory. We are using *C. elegans* to study AMPAR trafficking in an intact nervous system by visualizing localization of a chimeric protein containing the AMPAR subunit, GLR-1, fused to GFP (GLR-1::GFP). In the dendrites of wild-type animals, GLR-1::GFP is localized to discrete postsynaptic puncta. By contrast, mutants in *lin-10*, a Mint2 ortholog containing PTB and PDZ domains, accumulate GLR-1::GFP in large patches (1,2,3). In addition, *lin-10* mutants are deficient in their response to nose-touch mechanosensation, similar to *glr-1* mutant animals. To gain insight into the mechanism by which LIN-10 directs AMPAR trafficking, we used several genetic and cell biological approaches. First, using antibody labeling experiments that distinguish GLR-1 at the plasma membrane from intracellular GLR-1, we demonstrate that, in *lin-10* mutants, GLR-1 accumulates intracellularly. Second, by coexpressing GLR-1::GFP with RFP-tagged proteins that reside in distinct endosomal compartments, we show that excess GLR-1 accumulates in early endosomes in *lin-10* mutant animals. Third, we show that LIN-10 associates with these early endosomes, since GFP-tagged LIN-10 colocalizes with RFP::RAB-10, an early endosomal protein. Moreover, *rab-10* mutant animals display behavioral and GLR-1 localization phenotypes similar to those of *lin-10* mutants. Thus, we have identified RAB-10 as a novel regulator of AMPAR trafficking. Taken together, our data suggest that LIN-10 and RAB-10 function at synapses to mediate AMPAR trafficking out of early endosomes. (1) Rongo, C. et al. (1998) Cell: 94, 751-759. (2) Whitfield, C.W. et al. (1999) Mol Biol Cell: 10, 2087-2100. (3) Glodowski, D.R. et al. (2005) Mol Biol Cell: 16, 1417-1426.

556

**Role of Chaperone Proteins in KA2 Kainate Receptor Trafficking**

P. Vivithanaporn, G. T. Swanson; Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX

Glutamate receptor trafficking is controlled by interactions between chaperone proteins and specific trafficking motifs within receptor carboxy terminal domains. We found recently that intracellular retention of the ionotropic glutamate receptor subunit KA2, a member of the kainate receptor subfamily, is caused by the association of coatomer protein complex I (COPI) with an arginine-rich domain in the KA2 cytoplasmic domain. In a variety of other integral proteins, COPI competes for binding with 14-3-3 family proteins, which appear to promote plasma membrane expression. Here we tested the hypothesis that 14-3-3 and COPI proteins together regulate kainate receptor trafficking through competitive interactions with the carboxy terminal domain of the receptor subunit. We initially determined if KA2 subunits and 14-3-3 proteins were associated in a macromolecular complex after transfection of COS-7 cells with myc-KA2 cDNA. Myc-KA2 was detected in Western blots with anti-myc antibody following immunoprecipitation of the 14-3-3ζ isoform from cell lysates. Mutation of retention/retrieval determinants in the KA2 c-terminus, R862-6 and L908-9, reduced the interaction of homomeric KA2 receptors with COPI by 67% but increased interaction with 14-3-3ζ by 4 fold (n = 5). Mutation of R862-6 alone also increased the association of the receptor subunit with transfected VSVG-14-3-3ζ by 2 fold (n = 3) and increased plasma membrane expression by 5 fold as determined by cell ELISAs. Thus, increased 14-3-3ζ association was correlated with reductions in COPI binding and higher cell surface expression of the receptor. Homomeric KA2 receptors also interact with the 14-3-3β isoform, while neither GluR5-2b nor GluR6a subunits had detectable level of interactions with COPI or 14-3-3ζ proteins. These results indicate that competitive interactions between chaperone proteins modulate kainate receptor trafficking and targeting, which in turn control the availability of receptors at the cell surface.

557

**Glutamate Expression is Reduced by Melatonin in Cortex, Hippocampus and Brainstem of Rats with Induced Endotoxic Shock**

E. López-Robles, E. Meza-Lamas; Immunology, Universidad Autónoma de Zacatecas, Zacatecas, Mexico

Bidirectional communication between the immune systems and brain allow a regulation in response to infections. The endotoxic shock is caused by lipopolysaccharides (LPS) of gram-negative bacteria, followed by overproduction of pro-inflammatory cytokines; after arriving to the central nervous system (CNS) such cytokines modify neuro-endocrine and immune functions and stimulates the overproduction of neurotransmitters like glutamate. Melatonin (MLT) is a neuro-immunomodulator which exerts protective effects in septic shock and inflammation. We studied the role of melatonin in the glutamate expression in cortex, hippocampus and brainstem of rats with endotoxic shock induced by LPS (*E. coli* 0111:B4). Melatonin was administered in a course time in relation to LPS administration. Sprague-Dawley adult males 10/group received intraperitoneal injection as follows: (i) Control, 500 µl of normal saline. (ii) Control-MLT, 10 mg/body weight of melatonin (500 µl ethanol:saline). In the next groups 3µg/body weight of MK-801 was applicated 30 minutes before of $DL_{100}$ LPS (iii) Single injection of $DL_{100}$ LPS. (iv) LPS+MLT, dose of LPS and dose of melatonin simultaneously. (v) LPS+MLT/30, dose of melatonin 30 min after LPS. (vi) LPS+MLT/60, dose of melatonin 60 min after LPS. Brains were obtained at 90, 150 and 270 min after LPS, then cortex, hippocampus and brainstem were dissected and homogenized in sulphosalcilyc acid at 5%, then analyzed and compared with standard of amino acid glutamate by HPLC. Our results show that bacterial LPS up-regulates the amino acid glutamate in cortex and brainstem, furthermore the melatonin significantly decreased these levels. In conclusion, melatonin significantly decrease the glutamate production, in consequence melatonin would regulate the manifestations of endotoxic shock in CNS.

558

**Vesicular Glutamate Transporter T3 is Modulated in the Basal Ganglia of a Rat Model of Parkinson's Disease**

K. K. L. Yung, E. K. Y. Chung; Biology, Hong Kong Baptist University, Hong Kong, Hong Kong Special Administrative Region of China

Parkinson's disease is a serious motor disorder in human and it is caused by a degeneration of dopaminergic neurons in the substantia nigra pars compacta. One effect after dopamine denervation is an overactivity of glutamate pathways in the brain. The transport of glutamate into synaptic vesicles is mediated by a group of vesicular glutamate transporters, namely the VGLUT1 and VGLUT2, which are characterized and found in most of the population of glutamatergic synapses. Recently, a third vesicular glutamate transporter VGLUT3 has been identified and it is structurally and functionally related to VGLUT1 and T2. VGLUT3 protein was found to be expressed in cholinergic, glutamatergic, GABAergic as well as dopaminergic neurons in the basal ganglia. VGLUT3 protein is then suggested to play a role in Parkinson's disease. The objectives of the present project were to study the changes of expression of VGLUT3 in an animal model of Parkinson's disease, the 6-hydroxydopamine-lesioned rat. By immunocytochemistry, VGLUT3 immunoreactivity was seen to reduce in perikarya of GABAergic neurons in the globus pallidus of the lesioned side. In contrast, in the subthalamic nucleus of the lesioned side, VGLUT3 immunoreactivity was found to be increased in perikarya of neurons. In the substantia nigra pars reticulata, a significance increase in VGLUT3 immunoreactivity was also observed in perikarya of GABAergic neurons. The expression of VGLUT3 by GABAergic, cholinergic and dopaminergic neurons in basal ganglia raises the possibility that glutamate may be co-released with other neurotransmitters. However, the physiological role of VGLUT3 in these cells is still not clear. The modulation of VGLUT3 expression in basal ganglia found in the present study suggests that VGLUT3 may be involved in the etiology and pathogenesis of Parkinson's disease. Sponsor: Research Grants Council, Hong Kong HKBU2150/03M

559

**Glutamate Receptor Subunit Expression in a Mouse Model of Tuberous Sclerosis**

K. Cordero,[1] D. M. Talos,[2] F. Jensen[2]; [1]Biology, University of Puerto Rico at Mayaguez, Mayaguez, PR, [2]Neurology, Harvard Medical School, Boston, MA

Tuberous sclerosis complex (TSC) is a disorder which manifests epilepsy and mental retardation. The tumor suppressor genes, TSC1 and TSC2, are inactivated in TSC patients. Previous experiments showed an alteration in glutamate receptor subunits in human cortical tubers of TSC patients. These alterations may be involved in the excitability and seizures in TSC patients. We hypothesized that human pathology of TSC is similar to the animal model of TSC pathology. This study consists in a mouse model with the *Tsc1* gene inactivated with the purpose of keep investigating the role of abnormal glutamatergic transmission in TSC-related seizures. Animals without the mutation were used as controls. The experiments focused in looking for the abnormalities in cortical tubers and the alterations on the glutamate receptor subunits. Mice brains were cut at 50µm. Immunofluorescence was performed in TSC tissues and normal tissues. This technique was used for staining glutamate receptor subunits; NMDAR subtypes ( NR1, NR2A and NR2B ) and AMPAR subtypes ( GluR 1-4 ), abnormal neurons and glial cells and the mutation activation marker pS6. Immunostaining of the neurons and glial cells showed the presence of abnormal large cells in the cortex and hippocampus in mutant animals. These cells displayed positive staining for pS6, GluR1, NR1 and NR2B. Immunostaining of normal neurons expressed low immunorectivity for pS6 and exhibited GluR2, NR1 and NR2A subunits in wild type animals. Abnormal and large cells resembled dysplastic neurons of human cortical tubers in mutant animals. These cells were positive stained for PS6 and expressed the AMPAR and NMDAR subunits, GLUR1 and NR2B respectively, for Ca2+ permeability. The results of the immunostainined *Tsc1* animal tissue were consistent with the TSC human tissue.

560

**Effects of PKC-delta Inhibitor Rottlerin on $Na^+/K^+$ -ATPase Activity in Rat Brain Tissue *in vitro***

K. T. D. Nguyen,[1] J. Shin,[1] T. Knight,[1] R. Clarke,[2] P. Else,[3] V. J. Balcar[4]; [1]Anatomy and Histology, School of Medical Science, University of Sydney, Australia, [2]School of Chemistry, University of Sydney, Sydney, Australia, [3]Department of Biomedical Science, University of Wollongong, New South Wales, Australia, [4]Anatomy and Histology, Institute for Biomedical Research, University of Sydney, Australia

Experiments using protein kinase C (PKC)-delta inhibitor rottlerin have suggested that L-glutamate transport (GluT) could be regulated by PKC-delta mediated phosphorylation. However, rottlerin has also been shown to influence GluT by an unknown PKC-independent mechanism. Using deconvolution microscopy and advanced computer-assisted image analysis we have found that rottlerin indeed blocked the substrate- (D-aspartate-) induced movement (without causing any movement/redistribution of GLAST in the absence of the substrates) of glutamate transporter GLAST into the membranes of astrocytes cultured from neonatal rat brain. We have hypothesised that rottlerin inhibits $Na^+/K^+$-ATPase that normally provides energy for GluT. We have earlier shown that rottlerin can act as an uncoupler of oxidative phosphorylation in brain slices and this may result in an

apparent "inhibition" of $Na^+/K^+$-ATPase activity. Indeed, rottlerin significantly inhibited of $Na^+/K^+$-ATPase activity in both brain slices and cultured brain astrocytes as determined by $Rb^+$ uptake ($Na^+/K^+$-ATPase handles $Rb^+$ about equally well as $K^+$; $Rb^+$ was estimated by atomic absorption spectroscopy). Could a direct effect of rottlerin on $Na^+/K^+$-ATPase contribute to the inhibition of $Rb^+$ uptake? $Na^+/K^+$-ATPase activity was estimated by measuring inorganic phosphate released by cell-free preparations of actrocytic membranes and, using isolated non-neural membranes, by a fluorescence technique (dye RH421). Both methods have indicated that rottlerin is a potent inhibitor of $Na^+/K^+$-ATPase. Surprisingly, ouabain was only a weak inhibitor of $Na^+/K^+$-ATPase in actrocytic membranes. This could explain why ouabain, an inhibitor of GluT in brain slices, is often less effective in cultures of glial cells: expression of the most closely GluT-related ("ouabain-sensitive") $Na^+/K^+$-ATPase may vary from one preparation to another, depending on the particular culturing conditions. Inhibition of glutamate transport by rottlerin can, however, be mediated by the direct inhibitory effect of rottlerin on $Na^+/K^+$-ATPase(s).

561

**Histochemical Localization of Activin Receptors IA, IB, IIA and IIB on Normal and Stroked Brain Tissue**

K. C. Lopez; Biology, University of Puerto Rico, Mayaguez, PR

A stroke is a blockage or rupture of an artery that results in a lack of oxygen and glucose to the brain and, thus, death of brain cells. Stroke is a leading cause of death and disability in the US for which few therapies are available. Preliminary data from others in this laboratory suggest that a ligand called activin is produced by nerve cells and that they may increase after a stroke. This ligand is being studied because it is believed that it can minimize the amount of cell death during a stroke. Strokes were induced in mice using transient middle cerebral occlusion (MCAO). The expression of activin receptors IA, IB, IIA, and IIB was assayed using immunohistochemistry techniques to study and compare the location of the receptors on stroked and normal brain tissues. These studies are designed to test the hypothesis that neurons have activin receptors and that the amount of activin receptors will increase in the surroundings of the stroked area. The technique used to stain the tissue showed that all of the receptors mentioned earlier are present in the cortex. The identity of the cells with activin receptors will be explored using double label fluorescence and cell type specific antibodies.

562

**The Distribution of M1 and M3 Muscarinic Receptors and CB1 Cannabinoid Receptors at the Lizard Neuromuscular Junction**

K. A. Lewin, A. R. Graves, C. A. Lindgren; Biology, Grinnell College, Grinnell, IA

The current study used immunofluorescence to localize M1 and M3 muscarinic acetylcholine receptors (mAChRs) and type 1 cannabinoid (CB1) receptors at the lizard neuromuscular junction (NMJ). Previous work in our laboratory has shown that the activation of M1 mAChRs facilitates neurotransmitter release, while stimulation of M3 mAChRs inhibits release via the activation of CB1 receptors. We report that (1) M1 mAChRs are located on motor nerve terminals, perisynaptic Schwann cells (PSCs), and muscles; (2) M3 mAChRs are located on PSCs and muscles; and (3) CB1 receptors are located on nerve terminals and PSCs. Activation of M1 receptors in any of the three synaptic compartments may enhance neurotransmitter release, although activation of presynaptic M1 mAChRs provides the simplest explanation of M1-mediated enhancement. As for the M3-mediated inhibition, we propose that activation of postsynaptic M3 mAChRs causes the retrograde release of endocannabinoids, and the activation of presynaptic CB1 receptors ultimately inhibits neurotransmitter release. Interestingly, all receptor subtypes examined in this study were located on PSCs, suggesting a potential role for these cells in the muscarinic modulation of neurotransmission at the lizard NMJ.

563

**Microsporidian Spore Walls Equipped with Extracellular Nicotinic Acetylcholine Receptor(nAChR)-Gated Channel Proteins**

E. Weidner; Biological Sciences, Louisiana State University, Baton Rouge, LA

Microsporidian spores of the genus Spraguea are common nerve parasites in fish of the genus Lophius. We reported earlier (Pleshinger and Weidner, 1985) that Sprageua spores activate with a calcium ion influx. This calcium influx requires preconditioning with shift in extrasporular pH to the alkaline in the presence of polyanionic mucin. Micromolar concentrations of calcium antagonists lanthanum or verapamil blocked spore activation and discharge. Here we report the presence of nAChR alpha proteins in the outer spore envelope of Spraguea. This was determined by specific nAChR antibodies using immunolabeling and Western blots. In addition, nAChR alpha units were identified by fluorescent bungarotoxin. High resolution imaging indicates nAChR proteins confine to linear rows in the outer envelope of the Spraguea spore wall. The gated channels of Spraguea activate by a sudden pH shift in the presence of activator mucin. In the natural environs, spores attach to mucous glands in the anglerfish cirri (lower jaw). The spores enter mucous cells and discharge near adjoining neuronal junctions. It is essential the spores inject invasive sporoplasm directly into tthe target neuronal fibers.The primary target cells are supramedullary neurons known to extend fibers to the skin and mucous glands. Current questions: how do the nAChR proteins operate in the spore wall and are these proteins of host or parasite origin?

564

**Altered High Affinity Choline Uptake in Primary Cortical Neurons from Presenilin-1 M146V Mutant Knock-in Mice**

Q. Guo, D. J. Payette, J. Xie; Department of Physiology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK

The cholinergic neurotransmission plays a major role in regulation of many physiological functions. High affinity choline uptake is generally believed to be the rate-limiting step in acetylcholine synthesis in cholinergic neurons, and is essential for cholinergic transmission in the central nervous system. The high-affinity choline transporter-1 (CHT1) is a $Na^+$ and $Cl^-$-dependent, hemicholinium-3 (HC-3) sensitive choline transporter. Cholinergic hypofunction is associated with memory deficits in Alzheimer's disease (AD). Mutations in presenilin-1 (PS-1), which are causally linked to the pathogenesis of majority of familial AD cases, alter processing of the amyloid precursor protein (APP) in favor of production of the neurotoxic beta-amyloid peptide (Abeta), and increase neuronal vulnerability to apoptotic cell death. We previously found that pro-apoptotic proteins, such as Par-4, may alter CHT-1 mediated choline transport, resulting in a decreased choline uptake. We now report that high affinity choline uptake in primary cortical neurons from PS-1 M146V mutant knock-in mouse is significantly reduced compared to wild-type controls. Kinetic assays showed that the PS-1 mutation inhibited hemicholinium-3 (HC-3) sensitive choline uptake by reducing affinity of CHT1 to its substrate without significantly affecting the maximum levels of expression of CHT-1 in the plasma membrane. These results suggest that PS-1 mutations may contribute to the pathogenesis of dementia in AD by directly or indirectly affecting CHT-1 mediated choline uptake.

565

**EphB2 Receptor Signaling in Cultured Hippocampal Neurons involves Recruitment and Activation of FAK, Src, Paxillin, and RhoA**

M. L. Moeller,[1] Y. Shi,[1] L. F. Reichardt,[2] I. M. Ethell[1]; [1]Biomedical Sciences, University of California Riverside, Riverside, CA, [2]Physiology, University of California San Francisco, San Francisco, CA

The dendritic spines are the most common postsynaptic sites of excitatory synapses in the central nervous system and their formation is a critical aspect of synaptic development. The dendritic spine formation in hippocampal neurons is induced by synaptic contacts with presynaptic axon terminals and characterized by filopodia shortening and formation of mature mushroom-like spines, which accommodate the postsynaptic components of synapses. Defects in this process may underlie many neurodevelopmental disorders associated with mental retardation and autism. Previous studies by us and others have shown that interactions between EphB receptor tyrosine kinases and their cognate ligands, the ephrin-Bs, play a critical role in spine formation hippocampal neurons (Ethell et al., 2001; Penzes et al., 2003; Henkemeyer et al., 2003). Here we show a direct link between EphB receptor activation and intracellular signaling events associated with focal adhesion kinase (FAK). Activation of EphB2 receptors in cultured hippocampal neurons by ephrin-B2 results in the recruitment and assembly of a large, macromolecular protein complex resembling a focal adhesion complex (FAC). This complex includes FAK, Src, Grb2, and paxillin. Moreover, EphB2 activation leads to the additional activations of FAK, Src, and paxillin. Furthermore, the cre-mediated knockout of *loxP*-flanked *fak* or the siRNA-mediated disruption of FAK expression blocks EphB-mediated filopodia shortening. The results also show that EphB receptor activation induces RhoA activity suggesting that FAK/RhoGEF/RhoA mechanism might be responsible for the EphB-mediated shortening of dendritic filopodia. Based upon our current observations and past work, we speculate that downstream signaling events initiated by FAK might contribute to the EphB2-mediated formation of dendritic spines in hippocampal neurons. This work was supported by the NIMH grant MH67121.

566

**Axotrophin is Linked to LIF Signaling in the Nervous and Immune Systems**

G. E. Lyons,[1] M. A. Haendel,[1] P. A. D. Muthukumarana,[2] H. L. Thompson,[2] S. M. Metcalfe[2]; [1]Anatomy, University of Wisconsin Medical School, Madison, WI, [2]Surgery, University of Cambridge, Cambridge, United Kingdom

Axotrophin (axot) is highly conserved in mammals and the gene product has no clear homology with other proteins with the exception of a RING variant motif found in E3 ubiquitin ligase domains of the MARCH protein family. Discovered as a stem cell gene during gene trap mutagenesis screening, the axot null mouse revealed that axotrophin is involved in axonal regulation during formation of the corpus callosum in the brain and in the survival of unmyelinated C fibers of the substantia gelatinosa in the dorsal spinal cord. LIF mRNA levels are upregulated in the olfactory bulb and spinal cord of axot null mice. Unexpectedly, we found that axotrophin also plays a critical regulatory role in T lymphocyte responses, repressing proliferation (8-fold) and LIF release (5-fold). Analyses of isogenic T cell clones revealed phenotype-dependent effects, with Treg being associated with LIF release, in contrast to matched TH1 and TH2 effector T cell clones. In vivo, both axot and LIF expression were strongly associated with regulatory transplantation tolerance. This is the first evidence that LIF is regulated by axot and demonstrates new commonalities between embryonic stem cells and regulation of both the immune and nervous systems.

567

**Gonadotropin Receptor-Mediated Activation of G Protein-Activated Inwardly Rectifying $K^+$ (GIRK) Channels in Hypothalamic GnRH Neurons**

L. Hu, L. Z. Krsmanovic, K. J. Catt; Endocrinology and Reproduction Research Branch, National Institutes of Health, Bethesda, MD

Activation of luteinizing hormone (LH)/human chorionic gonadotropin (hCG) receptors in cultured hypothalamic cells and GT1-7 neurons transiently stimulates and subsequently inhibits cAMP production and pulsatile GnRH release. These changes were prevented by pertussis toxin (PTX), consistent with the coupling of LH/hCG receptors to adenylyl cyclase inhibitory G proteins. During LH treatment of both native and GT1-7 neurons, the frequency of spontaneous APs initially increased and progressively decreased during prolonged exposure. This inhibitory action of LH was also prevented by concomitant treatment with PTX, indicating that it is mediated by liberated $G_i/G_o$ subunits. Furthermore, GIRK1, 2, 3, and 4 channel subunits were found to be expressed in GT1-7 neurons. Whole-cell recording of GIRK current in normal and immortalized hypothalamic GnRH neurons was performed at 30 mM $K^+$ to increase current flow through GIRK channels, and channel activity was recorded as inward current at a holding potential ($V_H$) of -80 mV. The LH/hCG-induced currents were identified as GIRK currents based on their reversal potential of -30 mV, complete suppression by 200 μM $Ba^{2+}$, and inward rectification in the hyperpolarizing direction. During LH/hCG stimulation, maximum inward current was reached within 10-20 ms, followed by slow decay during prolonged stimulation. Pretreatment with PTX significantly reduced LH/hCG-induced GIRK current, indicating that this effect is mediated by liberated $G_i/G_o$ subunits. These data demonstrate that agonist-stimulated gonadotropin receptors in GnRH neurons activate GIRK channels, suppress membrane excitability, and slow spontaneous AP firing, leading to abolition of pulsatile GnRH release.

568

**Neuroglia Specific Expression of a FRET-based Calcium Indicator in Transgenic Mice**

S. D. Atkin,[1] L. A. Holtzclaw,[1] J. M. Pickel,[2] J. T. Russell[1]; [1]NICHD, NIH, Bethesda, MD, [2]NIMH, NIH, Bethesda, MD

Recent discoveries have highlighted the central role of glial cells in cellular signaling in the nervous system. Oligodendrocytes and Schwann cells receive signals during action potential traffic, and astrocytes monitor and modulate neural activity and plasticity. Such neuron-glial coordination relies on voltage and metabotropic calcium pathways and the mutual communications may play a vital role during development, maintenance of myelin, degeneration, and possibly regeneration of myelinated axons. We are interested in characterizing the calcium-based neuron-glial crosstalk in intact functioning brain tissue and peripheral nerve. One confounding problem is specifically labeling the glial cells of interest with appropriate $Ca^{2+}$ indicators such that physiological $Ca^{2+}$ signals can be recorded. We have developed transgenic mouse lines expressing the Cameleon (YC 3.60) $Ca^{2+}$ indicator protein under the control of a 9.6 kb human S100β glial-specific promoter. The promoter was fused upstream of the YC gene cassette. A sequence-verified clone of the plasmid was tested for cell specific expression by transfection and western analysis of C6 glioma and HEK cell cultures. Cellular $Ca^{2+}$ signals were measured in transfected C6 cells stimulated with 10μM ATP and up to a 90% increase in FRET was detectable. This construct was microinjected into C57Bl6/J embryos and founder mice and progeny were screened by PCR. Expression

characteristics were examined by immunohistochemistry using anti-GFP antibodies in 50µm brain sections from newborn pups. The transgene was expressed in 5 of 11 mouse lines. Expression is highly specific to astrocytes in the developing brain and CFP/YFP fluorescence is robust in two of the lines. Glial cell $Ca^{2+}$ signals associated with neural activity and axonal conduction can now be measured in the living animal, and these experiments are currently underway.

569

**Autoantibodies Associated with Lupic Psychosis Recognize a Novel Surface Protein and Induce Calcium Entry and Apoptosis in Brain Neurons**

S. Matus,[1] P. Burgos,[1] P. Farías,[2] O. Porras,[3] F. Torrealba,[2] F. Barros,[3] S. Jacobelli,[1] L. Massardo,[1] A. González[1]; [1]Depto. Reumatología. Fac. Medicina. Centro de Regulación Celular y Patología, Fac. Ciencias Biológicas. P. Universidad Católica de Chile, and MIFAB, Santiago, Chile, [2]Depto. Fisiología. Fac. Ciencias Biológicas. P. Universidad Católica de Chile, Santiago, Chile, [3]Centro de Estudios Científicos (CECS), Valdivia, Chile

More than fifteen years ago it was described that the psychosis of patients suffering from systemic lupus erythematosus (SLE) associates with autoantibodies against ribosomal P proteins (anti-P). This observation raised one of the most intriguing and controversial aspects in the pathogenesis of this prototypic autoimmune disorder. Anti-P autoantibodies recognize an epitope located in the carboxyterminus of three P ribosomal proteins, P0 (38 kDa) P1 (19 kDa) and P2 (17 kDa). In cells in vivo, intracellular antigens like ribosomal P proteins are normally inaccessible to circulating antibodies. There is evidence suggesting that these autoantibodies interact at the cell surface with a protein bearing the P-epitope. The antigenic target has not been identified but is currently believed it corresponds to the p38 ribosomal protein. The effect of these antibodies upon neuronal activity also remains completely unknown. We identify a novel transmembrane protein of high molecular mass (p>200) that exposes a ribosomal P epitope at the cell surface and is expressed exclusively by neurons at particular areas of the brain. Neurons in primary culture incubated with affinity purified anti-P autoantibodies rapidly increased their calcium levels and underwent apoptosis. Injection of anti-P antibodies into the brain also induced apoptosis in neurons. These results provide molecular and cellular bases supporting a pathogenic role of anti-P antibodies in neuropsychiatric lupus and open the possibility that dysfunctions of the p>200 protein might have pathogenic relevance in mental disorders in general. (Financial support from FONDAP 13980001 and the Millenium Institute for Fundamental and Applied Biology)

## Chloroplasts & Mitochondria (570-593)

570

**Transcriptional Regulation of Murine Propionyl Coenzyme A Carboxylase Beta Subunit Production**

K. Motejlek,[1] M. Koker,[2] C. Borowski[2]; [1]Gynecological Endocrinology, University Hospital, Aachen, Germany, [2]Institute of Pharmacology, University Hospital Eppendorf, Hamburg, Germany

The function of propionyl coenzyme A carboxylase (PCC) which is found abundantly in mammalian mitochondria is the ATP-dependent conversion of propionyl CoA into D-methylmalonyl CoA. The enzyme is composed of six alpha as well as six beta subunits and mutations in both subunit types result eventually in propionic acidemia in humans. PCC deficient patients show elevated plasma levels of propionic acid and glycine accompanied by symptoms like developmental delay, generalized seizures, coma, and death. Treatment usually includes lifelong dietary protein restriction for the patients. To get a better understanding of the transcriptional regulation of PCC beta subunit production we identified a 390 bp sequence within the full-length PCCB cDNA by deletion analyses that showed an enhanced basal transcription level compared to controls. This cDNA fragment contains a regulatory element of 83 bp in length at its 5'-end which is rich in purines. By bandshift mobility assays a single-stranded DNA-binding protein complex was revealed that showed specific binding to the upper DNA strand in competition assays. The protein complex consists of two subunits with molecular weights of roughly 28 and 42 kDa. As seen in SDS-PAGEs it is very likely that two of the smaller subunits are associated with one 42 kDa subunit. The binding sequence, identified by methylation interference, covers 12 bp in total. The contact site within the enhancer box consists of two identical face-to-face motifs of 5 bp each with 2 bp in between. Whether this single-stranded DNA-binding protein complex is sufficient to regulate PCC beta subunit production by itself or if additional proteins are required has to be determined. Also, any temporal or tissue-specific glycosylation and phosphorylation of this protein complex has yet to be examined. Supported by the University of Hamburg, Germany

571

**Thread-to-grain Transition of Mitochondria Induced by Cytomegalovirus-encoded vMIA is Independent of Pro-apoptotic Bcl-2 Family Member, Bax**

K. L. Norris,[1,2] M. Karbowski,[1] B. Dlouhy,[1,3] R. J. Youle[1]; [1]SNB, NINDS, NIH, Bethesda, MD, [2]Institute of Biomedical Sciences, George Washington University, Washington, DC, [3]Howard Hughes Medical Institute-NIH Research Scholars Program, Bethesda, MD

The *UL37* gene of human cytomegalovirus (HCMV) encodes the viral mitochondria-localized inhibitor of apoptosis, vMIA, which functions to inhibit apoptotic cell death. Apoptosis inhibition is an important virulence factor of HCMV. vMIA is known to bind Bax, a pro-apoptotic Bcl-2 family member. Bax is located in the cytosol of healthy cells but translocates to mitochondria upon induction of apoptosis. Paradoxically, vMIA binding of Bax and inhibition of apoptosis also results in Bax translocation to mitochondria. In addition, vMIA expression causes changes in mitochondrial morphology. Mitochondria exist in healthy cells in dynamic, highly connected networks, but expression of vMIA results in fragmentation of mitochondria into small, punctate units. The vMIA protein has two domains that are necessary and sufficient for the inhibition of apoptosis, a mitochondrial targeting sequence and a Bax-binding region. Since Bax is known to cause mitochondrial fragmentation during apoptosis, we examined the role of Bax in the vMIA-induced mitochondrial morphology rearrangement. Interestingly, we found that expression of vMIA in mammalian cells lacking endogenous Bax still resulted in fragmentation of mitochondria. Expression of Bcl-xl, an anti-apoptotic Bcl-2 family member that neutralizes Bax, was unable to reverse the fragmented phenotype. Expression of vMIA mutants lacking the Bax-binding function also led to the fragmented phenotype, whereas targeting of vMIA to mitochondria was required for mitochondrial fragmentation. Based on these results, we conclude that Bax binding is not required for fragmentation of mitochondria by vMIA. Future studies will focus on the purpose of the Bax-binding function, as well as the mechanism by which vMIA alters mitochondrial morphology.

572

**Two Critical Factors Affecting the Release of Mitochondrial Cytochrome c as Revealed by Studies Using *N,N'*-Dicyclohexylcarbodiimide as an Inducer of Permeability Transition**

T. Yamamoto,[1,2] S. Terauchi,[1,2] A. Tachikawa,[1,2] K. Yamashita,[3] M. Kataoka,[1] H. Terada,[4] Y. Shinohara[1,2]; [1]Institute for Genome Research, University of Tokushima, Tokushima, Japan, [2]Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima, Japan, [3]School of Dentistry, University of Tokushima, Tokushima, Japan, [4]Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda, Japan

Mitochondrial permeability transition (PT) causes inner mitochondrial membrane to become highly permeable. When PT is induced, acceleration of mitochondrial oxygen consumption and swelling of mitochondria are commonly observed. PT also causes the release of cytochrome c (cyt.c) from mitochondria, and these proteins trigger the following processes of apoptosis. However, the relationship between induction of PT and release of cyt.c from mitochondria has not yet been fully understood. *N,N'*-dicyclohexylcarbodiimide (DCCD), a chemical generally used for formation of peptide bond, had been reported to show stimulatory effects on the mitochondrial oxygen consumption and to induce swelling of mitochondria. These data seemed to imply that DCCD caused PT, but this possibility had never been investigated. In this study, to understand the relationship between induction of PT and release of cyt.c from mitochondria, we examined effects of DCCD on the mitochondrial structure and function in detail. As a result, DCCD induced mitochondrial swelling insensitively for cyclosporine A, known as a specific inhibitor of ordinary PT. Further addition of polyethylene glycol (PEG) solution to preswollen mitochondria caused shrinkage of mitochondria, reflecting the increased permeability of inner mitochondrial membrane. These results clearly indicated that DCCD induced PT in a cyclosporine A-insensitive manner. However, unlike many other PT inducers, DCCD failed to cause massive release of cyt.c. To understand the relationship between induction of PT and release of cyt.c, actions of DCCD on mitochondrial structure and function were compared with those of $Ca^{2+}$, known as an ordinary PT inducer. In conclusion, two parameters of mitochondrial volume and rate of mitochondrial oxygen consumption were considered as critical parameters controlling the release of cyt.c.

573

**Induction of Permeability Transition in Yeast Mitochondria Causes Release of Mitochondrial Cytochrome c**

A. Yamada,[1,2] T. Yamamoto,[2,3] M. Kataoka,[2] T. Nagata,[1] H. Terada,[4] Y. Shinohara[2,3]; [1]School of Dentistry, University of Tokushima, Tokushima, Japan, [2]Institute for Genome Research, University of Tokushima, Tokushima, Japan, [3]Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima, Japan, [4]Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda, Japan

$Ca^{2+}$ is known to elevate the permeability of mitochondrial membrane of mammals, and this transition is referred to as the mitochondrial permeability transition (PT). When mitochondrial PT is induced, the changes of mitochondrial structure and functions such as acceleration of mitochondrial oxygen consumption, swelling of mitochondria, and dissipation of mitochondrial membrane potential are commonly observed. PT also causes the release of cytochrome c (cyt.c) from mitochondria, and these proteins trigger the following processes of apoptosis. However, the mechanisms of release of cyt.c from mitochondria and induction of PT have not yet been fully understood. To understand the mechanisms causing mitochondrial PT or release of mitochondrial cytochrome c, genetic approach using yeast cells is useful. Previously, it was reported that PT could also be induced in yeast mitochondria. However, the question whether the PT induced in yeast mitochondria is associated with the release of mitochondriral cytochrome c is still uncertain. To understand the relationship between release of mitochondrial cytochrome c and induction of mitochondrial PT, in the present study, we examined functional properties of yeast mitochondria. When PT was induced in mitochondria isolated from yeast cells, acceleration of mitochondrial oxygen consumption, swelling of mitochondria, and dissipation of mitochondrial membrane potential were observed. Furthermore, release of cyt.c from mitochondria was observed when PT was induced in mitochondria isolated from yeast cells. These findings indicate that PT induced in yeast mitochondria is also associated with release of cyt.c from mitochondria as well as PT induced in mammalian mitochondria.

574

**Structural Change of the Cytosolic Second Loop of Mitochondrial ADP/ATP Carrier Revealed by Cysteine-scanning Mutagenesis**

A. Iwahashi,[1,2] E. Majima,[3] H. Terada,[4] Y. Shinohara[1,2]; [1]Institute for Genome Reserch, University of Tokushima, Tokushima, Japan, [2]Faculty of Parmaceutical Scinences, University of Tokushima, Tokushima, Japan, [3]APRO Life Science, Naruto, Japan, [4]Faculty of Parmaceutical Scinences, Tokyo University of Science, Noda, Japan

ADP/ATP carrier (AAC) catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane. Catalytic action of AAC is considered to be achieved by its interconversion between two distinct conformations, named c-state or m-state, in which substrate recognition site faces the cytosolic or matrix side, respectively. AAC is fixed into c-state or m-state by its specific inhibitors, carboxyatractyloside (CATR) or bongkrekic acid (BKA), respectively. Recently, crystal structure of bovine AAC complexed with CATR was reported. The revealed structure of AAC has six transmembrane segments connected by three and two loops facing to matrix and cytosol, respectively. However, it is still uncertain how AAC shows its catalytic activity on nucleotide exchange. To understand the catalytic mechanism of AAC, molecular characterizations of AAC at individual conformations are important. In the present study, to examine the structural properties of AAC at c- and m-state, we prepared various single-Cys yAAC2 mutants, having one particular Cys residue at the cytosolic second loop (LC2). These mutants were incubated with membrane-impermeable and fluorescent SH reagent, easin-5-maleimide (EMA), in the presence of CATR or BKA. By measuring reactivities of individual single-Cys yAAC2 mutant with EMA in c- and m-state, water-accessibility of Cys residue in LC2 was evaluated. As a result, conformation of LC2 changes between c- and m-state, suggesting that LC2 has important role as a gate.

575

**Intracellular Distribution of an Adenine Nucleotide Translocase in *Drosophila* Flight Muscles and the Challenge of Nucleotide Transport**

V. K. Vishnudas, S. S. Guillmette, J. O. Vigoreaux; Department of Biology, University of Vermont, Burlington, VT

Fast contracting muscles that experience high energy demands depend on diffusible ATP delivery mechanisms involving phosphagens. The *Drosophila* indirect flight muscles (IFM), however, do not appear to have a phosphagen based mechanism of ATP replenishment. Simple diffusion of nucleotides between the mitochondria (energy producer) and the myofibrils (energy consumer) is likely to maintain adequate ATP levels. In the myofibrils, ATP is hydrolyzed into ADP and Pi by myosin. Insect flight muscles are reported to exhibit high ATP flux (1000 μ mols $g^{-1}$ $m^{-1}$) during

flight. However the net flux of ADP by passive diffusion is low suggesting that other mechanisms may exists to facilitate the transport of ADP from myofibrils to mitochondria. Mass spectrometry analysis of *Drosophila* IFM skinned fibers revealed the presence of an adenine nucleotide translocase (ANT), encoded by the *stress sensitive B* (*sesB*) gene. Western blot analysis using an anti-bovine heart ANT antibody shows a single 32 kDa band in IFM skinned fiber samples. Immunofluorescence and quantitative immunoelectron microscopic studies suggest this ANT-like protein is associated with the myofibril. These results raise the possibility that an ANT shuttle system may be responsible for maintaining the flux of ATP and ADP into and out of the myofibrils. In order to address this possibility, we have made transgenic lines that express GFP tagged to the N terminus of two *sesB* cDNAs that differ in the length of their 3' Untranslated region (UTR). Mitochondrial proteins rely on cis elements in the 3' UTR of their mRNA for the correct localization of the mRNA and co translational import of the mature protein. The role of the different 3'UTRs in dictating the intracellular distribution of *sesB* is being examined by confocal microscopy.

576

**Function of StAR Signal Sequence is Controlled by its Pause**

M. Bose, D. Debnath, H. S. Bose; Department of Physiology and Funtional Genomic, University of Florida, Gainesville, FL

Steroidogenic acute regulatory protein (StAR), a 37kDa phosphoprotein, regulates steroidogenesis by facilitating cholesterol from the outer (OMM) to inner mitochondrial membrane (IMM). Mutant StAR cannot move cholesterol resulting in an inborn child disorder and fetuses die prematurely after birth. StAR, a 285-aminoacid protein, has a mitochondrial leader sequence, and cleaved to an intra mitochondrial 30kDa protein. To understand StAR's signaling process associated with its pause sequence, we hypothesized that deletion of its pause should increase its speed of entry into mitochondria and will not foster cholesterol movement, resulting no activity. Substitution of StAR leader (1-30 aa) or leader and pause together (1-62 aa) with mitochondrial matrix targeted protein, P450scc and its import into isolated mitochondria shows that StAR activity is proportional to its residency on the OMM. Signal sequence substituted StAR (Scc/N-30 StAR) imported slower than signal and pause (Scc/N-62 StAR) together. To characterize StAR-signaling mechanism in detail, we expressed Del-StAR (D31-62 aa) in E.coli, purified to homogeneity and characterized spectroscopically. Circular Dichroism analysis showed that Del-StAR is more a-helical at or below pH 4.5 and remains structured up to pH 1.0 without increasing coil. Partial proteolysis of Del-StAR with trypsin at pH 8.0 protected a band of 24 kDa, and with pepsin at pH 4.0 protected a ~5 kDa band. We found that partial proteolysis of biologically active N-62 StAR protected a 16 kDa band with Trypsin and Pepsin independently. Urea unfolding of Del-StAR at different pHs is 1.33 ±3 kcal/mol from pH 4.5 to 2.5, but it is 3.5 for N-62 StAR at pH 4.5 and no cooperativity of unfolding below pH 4.5. All these data suggest that the signal sequence changes StAR folding for its import.

577

**Comparison of Organellar Protein Profiles Between Hydrogen Peroxide Treated and Normal HeLa Cell Lines Using a Novel Technique for Separation and Enrichment of Organelles**

D. E. Kalman, J. A. Smith, H. Tran, W. Lan; Alfa Wassermann Proteomic Technologies, LLC, West Caldwell, NJ

Separation, fractionation and enrichment of subcellular components such as organelles is essential to study subcellular proteomes. Previously, differential and density gradient ultracentrifugation have been used to separate organelles. However, the efficiency and quality of these separation methods are limited by their batch mode and lengthy process time. In contrast to the traditional methods, we present a new technology: proteomics continuous-flow ultracentrifugation (pCFU$^{tm}$). pCFU provides a unique tool to separate and enrich organelles and organellar subtypes in one single and easy process. In this study, HeLa cells treated with hydrogen peroxide were compared to normal HeLa cell lines. Re-suspended cell pellets were dounce homogenized, followed by low-speed centrifugation to remove nuclei. pCFU was employed to further fractionate the supernatant and separate organelles by their buoyant density through a sucrose gradient. Mitochondria and ER enriched fractions were identified by Western blot analysis. To study the effects of hydrogen peroxide treatments on HeLa cells, the protein profiles of the organelle-enriched fractions from the treated cell lines were compared with those from normal HeLa cell lines using 2D gel electrophoresis and gel image analysis. Selected protein spots were digested with trypsin, and identified using nano-LC-ESI-MS/MS. Significant differences were observed between the protein profiles of the hydrogen peroxide treated HeLa cells and the normal HeLa cells. The results demonstrated that pCFU is a unique tool for fractionation, separation and enrichment of organelles and organellar sub-types. Consequently, pCFU technology in conjunction with 2D gel analysis significantly enhances subcellular proteomics studies.

578

**Mdv1 Interacts with Assembled Dnm1 to Trigger Mitochondrial Division**

K. Naylor,[1] V. Okreglak,[2] E. Ingerman,[1] J. E. Hinshaw,[3] J. Nunnari[1]; [1]Molecular and Cellular Biology, University of CA, Davis, Davis, CA, [2]Molecular and Cellular Biology, University of California, Berkeley, Berkeley, CA, [3]Laboratory of Cell Biochemistry and Biology, National Institutes of Health, NIDDK, Bethesda, MD

The dynamin-related GTPase, Dnm1, self-assembles into punctate structures that are targeted to the outer mitochondrial membrane where they mediate mitochondrial division. Post-targeting, Dnm1-dependent division is controlled by the actions of the WD repeat protein, Mdv1, and the mitochondrial TPR-like outer membrane protein, Fis1. Our previous studies suggest a model where at this step Mdv1 functions as an adaptor linking Fis1 with Dnm1. To gain insight into the exact role of the Fis1/Mdv1/Dnm1 complex in mitochondrial division, we performed a structure-function analysis of the Mdv1 adaptor. Our analysis indicates that dynamic interactions between Mdv1 and Dnm1 play a key role in division by regulating Dnm1 self-assembly.

579

**Mgr1p And Mgr3p Form a Substrate-Binding Subcomplex Within a Yeast Mitochondrial AAA-protease**

C. D. Dunn,[1] M. S. Lee,[2] F. A. Spencer,[3] R. E. Jensen[1]; [1]Cell Biology, Johns Hopkins School of Medicine, Baltimore, MD, [2]Genetics and Biology Center, National Heart, Lung, and Blood Institute, Bethesda, MD, [3]Institute of Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, MD

Mutation or depletion of the mitochondrial genome is associated with several human diseases, including cancer, diabetes, myopathy, and neurodegeneration. In contrast to most other eukaryotes, the yeast *Saccharomyces cerevisiae* can live without mitochondrial DNA (mtDNA). To

identify the genes that allow for the growth of cells containing impaired mitochondria, we performed a microarray-based, genome-wide screen for yeast knockouts that can no longer tolerate mtDNA loss. Among the many candidates that we identified were two novel genes that we call *MGR1* and *MGR3* (Mitochondrial Genome Required). Both Mgr1p and Mgr3p are located in the mitochondrial inner membrane and associate with Yme1p, the catalytic subunit of the i-AAA protease complex. We find that Mgr1p and Mgr3p are required for efficient degradation of misfolded substrates, and both proteins form a subcomplex that can bind i-AAA substrates even in the absence of Yme1p. Our work highlights the requirement for robust quality control at the mitochondrial inner membrane in cells lacking mtDNA. Studies are underway to identify the key substrates of the i-AAA protease and to pinpoint the mechanisms by which Mgr1p and Mgr3p facilitate proteolysis by the i-AAA complex.

580

**Expression Pattern of OPA1 Protein Isoforms in *Mouse* Brain**

V. R. Akepati,[1] A. Otto,[2] E. C. Müller,[2] C. Alexander[1]; [1]Neurodegeneration, Max-Delbruck-Centre for Molecular Medicine, Berlin-Buch, Germany, [2]Neuroproteomics, Max-Delbruck-Centre for Molecular Medicine, Berlin-Buch, Germany

Autosomal dominant optic atrophy (ADOA) is the most common form of hereditary optic neuropathies. Mutations in the OPA1 gene encoding a dynamin-related mitochondrial protein underlie ADOA and may perturb the biogenesis and maintenance of the mitochondria. The OPA1 protein is localized to the mitochondrial intermembrane space, where it is tightly bound to the outer leaflet of the inner membrane. It has eight mRNA isoforms as a result of the alternative splicing of exons 4, 4b and 5b. In this study we investigated various mouse tissues for specific expression of different OPA1 protein isoforms and their involvement in complex formation. We raised monoclonal antibodies against OPA1 isoform 1, bacterially expressed as a GST fusion protein. Western blot analysis of mitochondrial lysates with anti-OPA1 monoclonal antibody identified five bands of OPA1 in heart, liver, kidney and brain migrating around 100kDa. Certain OPA1 isoforms presented with higher abundance then others within one given tissue. In addition, comparison of the band patterns of the used lyastes revealed tissue specific expression levels of various isoforms. Immunoprecipitation of OPA1 from mouse brain mitochondrial lysate, followed by tandem MS/MS mass spectrometry analysis identified that isoform 1 and 7 were predominantly expressed in brain. We applied glycerol density-gradient ultracentrifugation to investigate complex formations by these different isoforms. The results indicate that isoforms of OPA1 form the different molecular weight complexes ranging from 200kDa. In parallel, we performed a high-throughput Yeast two-hybrid screen for various proteins expressed in brain that pulled out FEZ-1 (Fasciculation and elongation protein Zeta), Vim (Vimentin) and Hip5 (Huntington interacting protein 5) as possible interacting partners. Isoform 1 of OPA1 is predominantly expressed in brain, which forms a different molecular weight complex. Whether these complexes are composed as homo-oligomer or hetero-oligomer is yet to be investigated.

581

**Studies of the Putative Twin Pore Structure of the Mitochondrial Protein Import Complex TIM23 and the Essential Role of Tim17p**

S. M. Grigoriev,[1] S. Martinez-Caballero,[1] J. M. Herrmann,[2] M. Campo,[3] K. W. Kinnally[1]; [1]Basic Sciense, New York University College of Dentistry, New York, NY, [2]Institut für Physiologische Chemie, Universität München, München, Germany, [3]Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, Spain

Most mitochondrial proteins are encoded by the nuclear genome. Import of matrix-bound proteins across the mitochondrial outer and inner membranes is mediated by two distinct translocases, the TOM complex and the TIM23 complex. The single channel behaviors of these complexes were investigated using patch-clamp techniques. The roles of the TIM23 complex components Tim23p, Tim17p, and Tim44p in protein import and channel properties were analyzed using knockdowns and other mutants. While mitochondria depleted of Tim44p fail to import preproteins, they can import short peptides and the electrophysiological properties of the Tim23 channels are not altered. Import of preproteins and translocation of signal peptides into mitochondria is eliminated by depletion of either Tim17p or Tim23p. Tim23 channel activity is abolished by depletion of Tim23p. Interestingly, depletion of Tim17p eliminates the putative twin pore structure and distinctive voltage gating of the Tim23 channel. Studies of the effects of *Tim17* mutations reveal the essential role of the N-terminus in incorporation of Tim17p into the pore of the Tim23 channel, putative twin pore structure and voltage gating. We found that trivalent cations ($La^{3+}$ and $Gd^{3+}$) reversibly block both Tim23 and Tom channels suggesting these cations may interact with the preprotein's binding site(s) of the channels. Trivalent cations are now being used to probe Tim23 channels with modified pores e.g., Tim17ΔN. These studies support the notion that Tim23 channels have twin pores whose size and symmetry are modified directly or indirectly by Tim17p. (This research was supported by NSF grant MCB-0235834 and NIH grant GM57249 to KWK.)

582

**The Mitochondrial Outer Membrane Protein hFis1 Regulates Mitochondrial Fission and Apoptosis**

T. Yu, Y. Yong; Department of Anesthesiology and of Pharmacology and Physiology, University of Rochester, Rochester, NY

Mitochondrial fission is mediated by a defined set of protein factors and is involved in the early stage of apoptosis. In mammals, two proteins, the dynamin-like protein DLP1/Drp1 and the mitochondrial outer membrane protein hFis1, have been identified to participate in mitochondrial fission. The cytosolic domain of hFis1 contains six α-helices that form two tetratricopeptide repeat (TPR) motifs. To define the role of hFis1, we generated truncated and mutated hFis1 proteins and assessed mitochondrial morphology and DLP1 binding properties. We found that overexpression of α1-deleted hFis1 (hFis1[32-152]) induced aberrant swelling of mitochondria, which was reduced by Bcl-2 overexpression and inhibitors of mitochondrial permeability transition. We also found that the mitochondrial swelling induced by hFis1[32-152] required DLP1 binding to TPR motifs and was caused by increased binding of DLP1 to mitochondria. The co-expression of Bcl-2 with hFis1[32-152] restored the amount of DLP1 in mitochondria to the normal level. Interestingly, overexpression of the dominant-negative mutant DLP1-K38A had little effect on mitochondrial swelling induced by hFis1[32-152] although it markedly reduced mitochondrial fragmentation induced by full-length hFis1, indicating that the hFis1-DLP1 interaction is independent of the DLP1 GTPase activity. Co-expression of Bcl-2 with full-length hFis1 did not affect the mitochondrial fragmentation, whereas Bcl-2 expression reduced the mitochondrial swelling by hFis1[32-152]. These data suggest that Bcl-2 is not directly involved in the DLP1/hFis1-mediated mitochondrial fission but possibly in the early interaction of hFis1 and DLP1 during DLP1 recruitment. Additionally, we found that most of the swollen mitochondria by hFis1[32-152] retained cytochrome *c* up to 72 hours post-transfection, resulting in significant delay of apoptosis. Our data suggest that hFis1 is a main regulator of mitochondrial fission, controlling the recruitment and assembly of DLP1 during both normal and apoptotic fission processes.

583

**Photophysics of DASPMI Stained Mitochondria in Living Cells**

R. Ramadass, J. Bereiter-Hahn; Kinematic Cell Research Group;Bio-center, Johann Wolfgang Goethe University, Frankfurt, Germany

The styryl dye 2-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (DASPMI) is a low toxicity specific vital stain of mitochondria in living cells. Excitation and emission spectra, and quantum efficiency of DASPMI depend on solvent characteristics. Spectra of DASPMI stained mitochondria in living cells, correspond to those in a phospholipid environment (excitation around 470 nm, emission 560-570 nm). Fluorescence intensity of DASPMI is a dynamic measure for the membrane potential of mitochondria. In living cells, uptake of the dye is strongly influenced by uncouplers of oxidative phosphorylation such as CCCP (carbonylcyanide m-chlorophenylhydrazone). Photophysical properties of DASPMI were investigated using time- and space-correllated single photon counting technique. Spectrally and spatially resolved fluorescence decays of DASPMI stained mitochondria in living cells exposed to respiratory inhibitors are shown. Global analysis of the decays obtained showed a three exponential kinetics. The decay associated spectra of two of the three decay times were altered by different physiological conditions. These alterations on conditions reducing the mitochondrial membrane potential seem to be related to quenching processes (e.g. by energy transfer to oxidized cytochromes) and charge shifts in the mitochondrial membrane.

584

**Mitochondrial HDAC7 is Released from Inner Membrane Space Upon Apoptotic Insult**

R. E. Bakin, M. O. Jung; Radiation Medicine, Georgetown University Medical Center, Washington, DC

Control of global histone acetylation status is largely governed by the opposing enzymatic activities of histone acetyltransferases (HATs) and deacetylases (HDACs). HDACs were originally identified as modulators of nuclear histone acetylation status and have been linked to chromosomal condensation and subsequent gene repression. Accumulating evidence highlights HDAC modification of non-histone targets. Mitochondria were first characterized as intracellular organelles responsible for energy production through the coupling of oxidative phosphorylation to respiration. More recently, mitochondria have been implicated in programmed cell death whereby release of pro-apoptotic inner membrane space factors facilitates apoptotic progression. Here we describe the novel discovery that the nuclear encoded Class II human histone deacetylase HDAC7 localizes to the mitochondrial inner membrane space (IMS) of prostate epithelial cells and exhibits cytoplasmic relocalization in response to initiation of the apoptotic cascade. These results highlight a previously unrecognized link between HDACs, mitochondria, and programmed cell death.

585

**Aqp8 Water Channels and Mitochondrial Volume in Rat Liver**

G. Calamita,[1] D. Ferri,[2] P. Gena,[1] G. E. Liquori,[2] D. Thomas,[3] M. Svelto[1]; [1]Dept of General and Environmental Physiology, University of Bari, Bari, Italy, [2]Dept of Zoology, University of Bari, Bari, Italy, [3]UMR CNRS 6026, Rennes, France

Mitochondria are remarkably plastic organelles constantly changing their shape to fulfil their various functional activities. Although the osmotic movement of water into and out of the mitochondrion is central for its volume and activity, the molecular pathways for water transport across the inner mitochondrial membrane (IMM), the main barrier for molecules moving into and out of the organelle, are unknown. We recently reported an aquaporin water channel, AQP8, in rodent hepatocyte mitochondria (Ferri et al., Hepatology 2003; Calamita et al., JBC 2005). Here we employ immunoblotting, immunogold electron microscopy and stopped flow light scattering to investigate the correlations among mitochondrial AQP8 expression, mitochondrial morphology and water permeability. Both immunoblotting and immunogold electron microscopy localized AQP8 in the IMM of rat hepatocyte. The liver IMM was found to have a strikingly high coefficient of osmotic water permeability (Pf). Interestingly, the largest mitochondria featured the highest AQP8 expression and IMM Pf. AQP8 was also found in the mitochondria of other organs. The osmotic water transport of liver IMM was partially inhibited by the aquaporin blocker Hg2+, while the related activation energy remained low, suggesting the presence of a Hg2+-insensitive facilitated pathway in addition to AQP8. Overall, these results suggest that AQP8 facilitates the osmotic movement of water across the IMM. Moreover, the existence of an additional pathway other than aquaporins is postulated. AQP8 may be particularly important in the swelling which the mitochondrion undergoes following apoptotic stimuli and during oxidative phosphorylation. Recognition of AQP8 in mitochondria therefore has profound new implications for an understanding of how mitochondria adapt their volume.

586

**Dnm1, the Mitochondrial Division Dynamin, Forms Spirals that are Structurally Tailored to Fit Mitochondria**

E. Ingerman,[1] E. M. Perkins,[2] M. Marino,[3] J. A. Mears,[3] J. M. McCaffery,[2] J. Nunnari[1]; [1]Molecular and Cellular Biology, UC Davis, Davis, CA, [2]Department of Biology and Integrated Imaging Center, Johns Hopkins University, Baltimore, MD, [3]Laboratory of Cell Biochemistry and Biology, NIDDK, NIH, Bethesda, MD

Dynamin-related proteins (DRPs) are large, self-assembling GTPases whose common function is to regulate membrane dynamics in a variety of cellular processes. Dnm1, a yeast DRP (Drp1/Dlp1 in humans), is required for mitochondrial division, but its mechanism is unknown. Using a variety of biochemical techniques, we identified two novel regulatory features of Dnm1. Dnm1 self-assembly proceeded through a rate-limiting nucleation step and nucleotide hydrolysis by assembled Dnm1 structures was highly cooperative, with respect to GTP concentration. As seen by electron microscopy, Dnm1 formed extended spirals, which possessed diameters greater than those of the prototypic DRP, dynamin-1. The diameters of the Dnm1 spirals, remarkably, were equal to those of *in vivo* mitochondrial constriction sites, which we visualized by electron microscopy in thin sections of yeast cells. Thus, our data support a model in which nucleation-dependent self-assembly of Dnm1 into spirals drives the constriction of the outer and inner mitochondrial membranes during mitochondrial fission. Our data further suggest that Dnm1 has evolved to form structures that fit the dimensions of mitochondria.

587

**A Mitochondrial DNA Analysis of the Major Ethnic Groups in Sierra Leone and Cameroon**

J. L. Wilson,[1] B. Ely,[2] B. A. Jackson[3]; [1]Department of Chemistry, University of Massachusetts Lowell, Lowell, MA, [2]Department of Biological Sciences, University of South Carolina, Columbia, SC, [3]Biomedical Engineering and Biotechnology Program, Department of Work Environment,

University of Massachusetts Lowell, Lowell, MA

Previous studies of African human mitochondrial DNA (mtDNA) have focused primarily on discerning the origins of modern humans and the worldwide expansion of human populations. These same studies have demonstrated that Africa contains the greatest level of mtDNA diversity in the world (Ingman et al., 2000). However, there has been no published study to characterize genetic differences in the mtDNA of the many ethnic groups of Africa that were the sources of slaves for the Americas and Caribbean. Such a study would be important to blacks of these regions whose African heritage was lost due to slavery. The hypervariable one region of mtDNA from 276 individuals from Sierra Leone and 650 individuals from Cameroon of various ethnic groups were sequenced and haplogroups determined. An analysis of molecular variance indicated that the distribution of these haplotypes within certain ethnic groups was significantly different from that of the other ethnic groups. The results of this study indicate that distinguishing genetic differences can be observed among African ethnic groups residing in historically close proximity to one another (Jackson et al., 2005). Furthermore, it was observed that some mtDNA haplotypes are common among the Sierra Leone and Cameroonian ethnic groups, but have not been observed in other published studies of West African ethnic groups. Therefore, this may be the first genetic evidence for mtDNA lineages that are unique to these regions of West Africa.

588

### Analysis of *Gregarines niphandrodes* Extranuclear DNA

M. Toso, C. K. Omoto; School of Biological Sciences, Washington State University, Pullman, WA

We investigated whether or not gregarines possess extranuclear DNA and if so, their identity. Gregarines are understudied unicellular parasites of invertebrates in the phylum Apicomplexa. Many apicomplexa possess two types of extranuclear DNA, mitochondrial and plastid. The non-photosynthetic plastids for a number of apicomplexans (*Plasmodium, Toxoplasma, Babesia, Eimeria, Theileria*) contain 35kb circular genome. The apicomplexan mitochondrial genome is unusual in being small, 6kb, and linear. It encodes two proteins: cytochrome C and cytochrome B and small fragments of rRNA gene. Whole genome sequencing of one genus of apicomplexa, *Cryptosporidium*, shows that it lacks both the plastid and mitochondrial genomes. Phylogenetic analyses using both 18S ribosomal RNA gene, and DNA-dependent RNA polymerase, largest subunit (RPB1) indicates that gregarines and *Cryptosporidium* are closely related. This relationship raises the question, "Do gregarines possess extranuclear DNA?" 4',6-Diamidino-2-phenylindole (DAPI) staining of *Gregarina niphandrodes* trophozoites shows extranuclear punctate staining. PCR using plastid-specific primers that amplified LSU rRNA and *tufA*-tRNA sequences from *Plasmodium falciparum* and *Toxoplasma gondii* did not produce any amplicons with *Gregarina niphandrodes* DNA. We did not observe an organelle with plastid ultrastructure in transmission electron microscopy of *Gregarina niphandrodes* trophozoites, yet mitochondria were observed. Using primers for the mitochondrial cytochrome C gene, we have retrieved a partial sequence of the gene from *Gregarina niphandrodes*. Phylogenetic analysis of this sequence indicates that it is a member of the apicomplexan mitochondrial cytochrome C gene family. Therefore, we conclude that *Gregarina niphandrodes* does not possess either a plastid organelle or a plastid genome. However, *Gregarina niphandrodes* possesses extranuclear DNA and it is mitochondrial. We are currently completing the sequencing of this mitochondrial genome.

589

### Regulation of Mitochondrial Morphology by Intracellular $Ca^{2+}$

J. R. Hom,[1] S. Sheu,[1] Y. Yoon[2]; [1]Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY, [2]Anesthesiology, University of Rochester Medical Center, Rochester, NY

Mitochondria are dynamic organelles that frequently change shape and location within the cell. This dynamic behavior is mediated by defined sets of factors, including proteins involved in fission, fusion and translocation of mitochondria. While many protein factors directly participating in mitochondrial dynamics have been identified, upstream signals that regulate mitochondrial morphology are not understood. In this study, we tested the role of intracellular $Ca^{2+}$ in regulating mitochondrial morphology. We found that mitochondria underwent two phases of fragmentation upon exposure to the ER $Ca^{2+}$-ATPase inhibitor thapsigargin (TG). The initial fragmentation of mitochondria was a transient event, as mitochondria became rapidly fragmented within 10 minutes following the cytosolic $Ca^{2+}$ transient and tubular mitochondria were restored in an hour after cytosolic $Ca^{2+}$ decreased to the resting level. We found that the initial fragmentation of mitochondria is $Ca^{2+}$ concentration-dependent, suggesting that it is likely mediated by cytosolic $Ca^{2+}$ signaling. In addition, inhibition of the mitochondrial fission protein DLP1 blocked the fragmentation, indicating that TG-induced mitochondrial fragmentation occurred via mitochondrial fission process. Furthermore, inhibition of hFis1, the upstream DLP1 recruiting protein, also prevented the TG-induced mitochondrial fragmentation, suggesting that increased cytosolic $Ca^{2+}$ acts at the early stage to activate cellular mitochondrial fission machinery. We also found that prolonged incubation with TG caused non-reversible apoptosis-associated mitochondrial fragmentation. In contrast to the initial fragmentation, the second phase mitochondrial fragmentation occurred regardless of TG-induced cytosolic $Ca^{2+}$ increase, suggesting that sustained TG treatment caused ER stress-mediated apoptosis through ER $Ca^{2+}$ depletion. We also identified a potential role of DLP1 in mitochondrial outer membrane permeabilization that leads to cytochrome *c* release in the absence of mitochondrial fission during TG treatment. Our data demonstrate that $Ca^{2+}$ participates in controlling mitochondrial morphology via intracellular $Ca^{2+}$ signaling as well as apoptosis-related process.

590

### The Opa1 (optic Atrophy 1) Gene Is Essential for Embryonic Development in the Mouse

M. Fiket,[1] M. Seeliger,[2] C. Alexander[1]; [1]Neurodegeneration, Max-Delbrueck-Center for Molecular Medicine, Berlin-Buch, Germany, [2]Retinal Electrodiagnostics Research Group, Dept. of Pathophysiology of Vision and Neuroophthalmology, University Eye Hospital, Tuebingen, Germany

*OPA1* is a nuclear gene encoding a large mitochondrial dynamin-related GTPase implicated in mitochondrial fusion and maintenance. Mutations affecting the coding region of *OPA1* result in the clinical phenotype of autosomal dominant optic atrophy (adOA), which leads to visual loss in humans and retinal ganglion cell death. We sought to generate a mouse model allowing us to analyse OPA1 function in vivo. Most of the disease causing mutations in adOA patients result in haploinsufficiency. Therefore, we disrupted exon 2 of the mouse *OPA1* gene by introducing a Neomycin cassette at the exon/intron border. Our targeting strategy completely abolished functional gene expression from this *mOPA1* allele, theoretically resulting in a dosage effect in heterozygous animals, and loss of function in homozygous m*OPA1*-knock-out mice. Heterozygous mice are viable and fertile. We have examined eyes and optic nerves of such mice at different stages of postnatal development. Plastic sections of retinae

and optic nerves were stained with cresyl violet. Electroretionography (ERG) and scanning laser ophthalmoscopy (SLO) were performed in order to test for functionality of various retinal cell types and retinal ganglion cell properties. Unfortunatelly, we could not detect phenotypic changes in the visual system between *mOPA1*-deficient and wildtype mice. Homozygous mutants are embryonic lethal and die between e7.5 and e8.5. We conclude that, in contrast to humans, mice seem to be able to compensate for the loss of a functional *mOPA1* gene copy. On the other hand, embryonic lethality of homozygous mutants reveals that humans, as well as mice , greatly depend on OPA1 function. Further examination of those very early dying embryos will allow us to understand this unexpectedly harsh phenotype and the essential involvement of OPA1 in mitochondrial function.

591

### Targeting Of Rice Glycoprotein Alpha-amylase I-1 Into Plastid: Plastid Targeting Signal

T. Mitsui,[1,2] S. Asatsuma,[3] C. Sawada,[3] A. Kitajima[3]; [1]Dept. Applied Biol. Chem., Niigata University, Niigata, Japan, [2]Lab. Plant Microbial Genome Control, Niigata Univ., Niigata, Japan, [3]Lab. Plant Microbial Genome Control, Niigata University, Niigata, Japan

Previous our study has shown that alpha-amylase I-1 is involved in degradation of plastidial starch in rice living cells (Plant Cell Physiol. 46:858-869, 2005). In the present study, we performed transient expression and targeting of rice alpha-amylase I-1 fused with green fluorescence protein (GFP) in onion epidermal cells. It was found that alpha-amylase I-1-GFP fusion protein targets into the plastids visualized with the plastidial marker WxTP-DsRed in bombarded onion cells. In contrast, the other alpha-amylase isoforms (II-4, II-5, and II-6) did not localize in the plastids. These results indicate that rice alpha-amylase I-1 contains a plastid targeting signal common to both monocotyledonous rice and onion cells. To determine an important region for the plastidial targeting of alpha-amylase I-1, transient expression and localization of a series of carboxy-terminal truncated native alpha-amylase I-1-GFP fusion protein was tested. The results showed that the peptide region from 301 to 369 amino acid residues including a putative starch binding site is important for plastid targeting of alpha-amylase I-1 in onion cells. We further examined the targeting characteristics of the starch binding site-mutated alpha-amylase I-1-GFP fusion protein in onion cells. The Typ301-Typ302 was a unique amino acid residues in the polypeptide of alpha-amylase I-1, while the other alpha-amylase isoforms, such as II-4, II-5, and II-6, had no the tryptophan dipeptide in this area. The substitution of Ala302 for Typ302 strongly arrested the targeting of alpha-amylase I-1-GFP into the plastids. Based on these overall results, we concluded that the Typ302 residue is necessary for the plastid targeting of alpha-amylase I-1 in onion epidermal cell.

592

### The Outer Envelope of Plant Plastids Functions as Selective Permeability Barrier for Solutes During Plant Development

K. Philippar, A. Timper, R. Thomson, J. Soll; Department for Biology I, Botany, Ludwig-Maximilians-University of Munich, Munich, Germany

Plastids of higher plants originate from the endosymbiosis of a cyanobacterium with an early eukaryotic cell. In the green organs of plants this leads to the evolution of chloroplasts, representing the site of photosynthesis. However, chloroplasts are only one member of the organelle family called plastids. Besides photosynthesis, plastids conduct specialised functions that are essential for plant growth and development (e.g. nitrogen assimilation, storage of carbohydrates and lipids, synthesis of amino acids, fatty acids and secondary compounds). Because of this fundamental biosynthetic capacity, function of the plant cell requires the exchange of metabolites between plastids and the surrounding cytosol. As their Gram-negative ancestors, plastids are delimited by two membranes, the outer and inner envelope. Communication in this integrated metabolic network is thus mediated by transport proteins in the envelope membranes. Due to the presence of porins, the outer envelope has been assumed to be a size-selective sieve only, whereas the inner envelope has been considered to be the actual permeability barrier. However, several channel-like proteins, exhibiting substrate specificity and characteristic gating properties could be isolated from chloroplastic outer envelopes of pea. To prove the concept of a regulated gate in the outer envelope of plastids, we characterized mutants of OEPs ("outer envelope proteins") in the genetic model plant *Arabidopsis thaliana.* Here the OEP24 family shows specific expression pattern in developing seeds and pollen. While a T-DNA insertion mutant of *OEP24.1* is homozygous-lethal and impaired in pollen germination, expression of *OEP24.2* is high in late stages of seed development and in germinating seeds. Therefore, OEP24.1 functions as an essential and selective transport barrier in the outer envelope of plastids, supplying solutes for early pollen development. In contrast, OEP24.2 operates in the storage-type plastid of seeds.

593

### Syntabulin-Mediated Anterograde Transport of Mitochondria Along Neuronal Processes

Q. Cai, Z. Sheng; Synaptic Function Unit, National Institute of Neurological Disorders and Stroke, National Institute of Health, Bethesda, MD

In neurons, proper distribution of mitochondria in axons and at synapses is critical for neurotransmission, synaptic plasticity, and axonal outgrowth. However, mechanisms underlying mitochondrial trafficking throughout the long neuronal processes have remained elusive. Syntabulin is originally identified by our lab and implicated in the syntaxin-1 transport in neurons (Nat. Cell Biol. 2004, 6:941-953). Here, we report that syntabulin plays a critical role in mitochondrial trafficking in neurons. Syntabulin is a peripheral membrane-associated protein which targets to mitochondria through its carboxyl terminal tail. Using real-time imaging in living cultured hippocampal neurons, we demonstrate that a significant fraction of syntabulin co-localizes and co-migrates with mitochondria along neuronal processes. Knockdown of syntabulin expression with targeted siRNA or interference with the syntabulin-kinesin-1 heavy chain interaction with dominant negative transgenes reduces mitochondrial density within axonal processes by impairing anterograde movement of mitochondria. These findings collectively suggest that syntabulin acts as a linker molecule capable of attaching mitochondrial organelles to the microtubule-based motor kinesin-1, and in turn, contributes to anterograde trafficking of mitochondria to the neuronal processes.

## Endoplasmic Reticulum (594-615)

594

### Regulation of the Unfolded Protein Response by Small GTP Binding Proteins in Celegans

M. Caruso,[1] S. Jenna,[1] M. Vidal,[2] D. Baillie,[3] E. Chevet[1]; [1]McGill University, Montreal, PQ, Canada, [2]Harvard Medical School, Boston, MA, [3]Simon Fraser University, Vancouver, BC, Canada

The Unfolded Protein Response (UPR) is a conserved adaptive mechanism activated in eukaryotic cells subjected to physiological stresses. It is

caused by the accumulation of mis/unfolded proteins in the endoplasmic reticulum (ER) which in turn promote the activation of ER resident sensors and their downstream signaling cascade. This leads to three major outcomes including translation inhibition, activation of the SAPK/MAPK pathway and activation of specific transcriptional programs. In an attempt to characterize novel regulators of the UPR, we have developed a systematic gene silencing approach with *Caenorhabditis elegans* as a model system. Our efforts have at first focused on small GTP binding proteins which are key regulators of the exocytic, endocytic and signal transduction pathways. Therefore, we postulated that an alteration of the expression of these GTPases could impact on the UPR in *C.elegans*. To visualize this phenomenon, we are studying twelve *C.elegans* GFP reporter strains for genes whose expression is potentially UPR-regulated in the worm. We observed that most of these reporters show variable level of expression but are mainly expressed in the intestine under basal conditions. When exposed to the ER stressor tunicamycin, five strains showed a significant increase in GFP expression at both qualitative and quantitative levels. Consequently, from the 60 GTPases encoding genes identified in *C.elegans* genome, we selected only those for which the expression product localized in the intestine and co-localized with compartment markers of the secretory pathway. This strategy resulted in the selection of only one third of the initial GTPase population. These selected targets are systematically silenced in the five reporter strains in the presence or absence of tunicamycin. Preliminary observations revealed that the Rab family of GTPases may represent a novel complex regulator of the Unfolded Protein Response.

595

**Xbp-1(s) Contributes to Ig Heavy Chain Expression at Both Transcriptional and Post-transcriptional Levels in a Plasmacytoma Cell Line**

Y. Shen,[1] Y. Jin,[1,2] L. M. Hendershot[1,2]; [1]Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, [2]Department of Molecular Sciences, University of Tennessee, Health Science Center, Memphis, TN

ERdj3/HEDJ is a component of unassembled immunoglobulin heavy chain (HC):BiP multi-chaperone complexes and is highly expressed in HC only plasmacytoma cell lines as compared to $Ig^-$ cell lines. Recently ERdj3 was shown to be a target of the XBP-1 transcription factor, which is induced (via ATF6) and spliced (via Ire1) during UPR activation. The spliced form of XBP-1 is required for plasma cell differentiation, thus raising the possibility that XBP-1 contributes to HC stability in plasma cells. In this study, we demonstrate that although XBP-1 is responsible for the up-regulation of ERdj3 during ER stress, it did not control basal expression of ERdj3 under normal physiological conditions in non-lymphoid cells. Thus, we wished to determine if XBP-1(S) contributed to high steady state ERdj3 levels in HC only cells or if instead this represented an increased basal expression of ERdj3. We found that XBP-1(S) was highly expressed in plasmacytoma cells that produce unassembled HCs, indicating that the UPR remains active in a stable plasmacytoma line. When XBP-1 expression was knocked down in the plasmacytoma cells, the expression of *ERdj3* was modestly compromised. Surprisingly, the transcription of BiP was reduced as well both in the absence and presence of the HC. This indicates that XBP-1(S) contributes to the expression of ERdj3 and BiP in plasma cells, and that this regulation is independent of antibody production. Expression of HC was also diminished, but unexpectedly this occurred at the transcriptional level. Our data revealed that XBP-1(S) positively regulated the HC enhancer gene OBF1/BOB1, thus serving to increase HC expression at the transcriptional level.

596

**The C-Terminal Domain of Calreticulin (CRT) Mediates Retrotranslocation to the Cytoplasm Independently of ER-associated degradation**

N. Afshar,[1] B. E. Black,[2] B. M. Paschal[1]; [1]Center for Cell Signaling, University of Virginia, Charlottesville, VA, [2]University of California, San Diego, San Diego, CA

CRT is a calcium-binding chaperone that undergoes synthesis at the endoplasmic reticulum (ER). However, CRT has been implicated in multiple nuclear and cytoplasmic processes, including cell adhesion, RNA binding, transcriptional regulation, and nuclear transport. In contrast to the dogma that CRT influences these processes from within the ER lumen, we have advanced the alternative view that CRT plays a direct role in these processes by undergoing retrotranslocation to the cytoplasm. We previously used a battery of assays to show that CRT retrotranslocates out of the ER lumen (MCB in press). Here we determine the domain of CRT that mediates retrotranslocation. We targeted an artificial transcription factor (ERX) to the ER lumen with an N-terminal signal sequence. Fusing CRT to ERX enables the transcription factor to escape the ER lumen, and, subsequently, enter the nucleus where it activates a luciferase-based reporter plasmid. Retrotranslocation activity was mapped to the C-terminal region of CRT, which was both necessary and sufficient for retrotranslocation. The latter conclusion was based on the ability of the CRT C-domain to mediate retrotranslocation of PDI. To date, retrotranslocation has been associated with ER-associated degradation of misfolded proteins. CRT retrotranslocation, however, is not dependent on proteasome activity, nor does it involve ubiquitylation. As CRT has been implicated in cell adhesion through its interaction with integrins, we examined the adhesion and motility properties of *crt-/-* mouse embryonic fibroblasts (MEFs). *crt-/-* MEFs were reduced in cell migration two-fold relative to WT MEFs. *crt-/-* MEFs also displayed reduced binding to collagen, which was rescued by transfecting a form of CRT that is not targeted to the ER. CRT is the first example of a protein that relies on retrotranslocation to control its compartmentalization in the cell.

597

**Autophagy is Activated During ER Stress via the ER-resident Transmembrane Kinase IRE1**

M. Ogata,[1,2] F. Urano,[3] N. Mizushima,[4] K. Imaizumi[1]; [1]Faculty of Medicine, University of Miyazaki, Miyazaki, Japan, [2]Graduate School of Biological Sciences, NAIST, Nara, Japan, [3]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, [4]Department of Bioregulation and Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

The endoplasmic reticulum (ER) is the compartment in which protein folding occurs prior to transport to various intracellular organelles. A number of cellular stress conditions lead to the accumulation of unfolded or misfolded proteins in the ER lumen, which constitutes a fundamental threat to the cell and triggers the unfolded protein response (UPR) to avoid cell damage. The UPR involves at least three distinct components, namely transcriptional induction of genes encoding ER-resident chaperones to facilitate protein folding, translation attenuation to decrease the demands made on the organelle and ER-associated degradation (ERAD) to degrade the unfolded proteins accumulated in the ER. Despite of extensive characterization of the signaling pathway for the UPR during ER stress, the morphological changes after ER stress are not well understood. Therefore, we performed electron microscopical examination of cells that were exposed to ER stress. In SK-N-SH neuroblastoma cells, treatment with tunicamycin, DTT, and thapsigargin induced the formation of autophagosomes, which were recognized at the ultrastructural level as double-

membrane vacuolar structures containing visible cytoplasmic contents. Immunoblot analysis revealed that the activated form of LC3 (LC3-II) is increased by ER stress, indicating that ER stress activates autophagy systems. In IRE1α-deficient cells, the formation of autophagic vacuoles induced by ER stress was inhibited. This result showed that IRE1α is essential for autophagy activation after ER stress. Furthermore, cell death induced by ER stress was accelerated by treatment with 3MA, a classical inhibitor of autophagy, suggesting that autophagy plays important roles in cell survival after ER stress.

598

**Cold Adaptation in Yeast: the Role of ER-associated Degradation and Sterol Biosynthesis**

R. Wright,[1] A. Felthauser,[1] J. Merkel,[1] A. Sturm,[2] M. Bard[2]; [1]Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN, [2]Department of Biology, Indiana University-Purdue University Indianapolis, Indianapolis, IN

In a search for genes involved in membrane biogenesis in yeast, we discovered an unexpected link between cold adaptation and the endoplasmic reticulum (ER) quality control system known as ERAD (ER-associated degradation). Through the process of ERAD, short-lived, misfolded, or unassembled proteins are ubiquitinated and degraded by the proteasome. In a subset of ERAD mutants (*ubc7, cue1,* and *doa10)* expression of increased HMG-CoA reductase results in cold-sensitivity, such that the mutant strains are unable to grow at temperatures of 16°C or lower. In addition, the *UBC7, CUE1* and *DOA10* genes are required for growth at temperatures of 10°C or lower. Analysis of the sterol composition of these cold-sensitive mutants provides a possible mechanistic explanation of the relationship between ERAD, sterol metabolism, and growth at cold temperatures. In the mutant strains, the overall sterol composition is altered, such that the mutants contain decreased levels of squalene and increased levels of lanosterol. These results suggest that a primary defect produced by loss of *UBC7, CUE1,* or *DOA10* function is the inability to properly regulate sterol metabolism. In turn, this altered sterol composition may lead to cold sensitivity. Consistent with this hypothesis, loss of any non-essential gene required for sterol biosynthesis in yeast (*ERG2, 3, 4, 5, 6 24,* and *28)* results in cold-sensitivity. Thus, regulation of sterol biosynthesis appears to be critical for cold adaptation in the yeast, and ERAD plays a role in that regulation.

599

**The Role of IRE1's Kinase Domain during the Unfolded Protein Response**

A. Chawla, M. Niwa; Biology, UCSD, La Jolla, CA

The unfolded protein response (UPR) is a stress response pathway, sensing the accumulation of unfolded proteins in the endoplasmic reticulum (ER), and relaying this information to the nucleus. In yeast, IRE1, an ER resident transmembrane receptor kinase/ endoribonuclease, senses the presence of unfolded proteins. Rising levels of unfolded proteins, lead to IRE1's oligomerization and kinase activation. Activated IRE1 then functions as the site-specific endoribonuclease, cleaving a unique intron from HAC1 mRNA. The spliced form of HAC1 mRNA codes for a potent UPR specific transcription factor, which turns on the expression of ER chaperones and protein folding enzymes, ultimately allowing for the re-establishment of ER protein homeostasis, and turning off the IRE1 nuclease. We have found a novel IRE1 mutant which seems to be impaired in the recovery phase. In wild-type cells, HAC1 splicing, peaks at ~2hrs (60% spliced HAC1), after which it starts to decline, suggesting recovery from ER stress. In the case of the mutant however, HAC1 splicing reaches ~80% of wild-type levels by 2hrs, and is sustained for an extended amount of time (upto 8hrs). To examine if ER stress is sustained, even after HAC1 splicing, in cells carrying the mutant IRE1, we over-expressed one of the major ER chaperones, KAR2, but this did not help the mutant IRE1 to recover faster. In addition, increasing HAC1 splicing in the mutant cells to the level similar to wild-type, did not aid in recovery of the mutant. Together, these observations suggest that the sustained splicing observed in the mutant cells results from the inability of IRE1 to turn off its nuclease. We are currently investigating the molecular bases of the mechanism of shutting off IRE1.

600

**Dynamic Association of Rab and Arf GTPases with Adiposomes (Lipid Droplets)**

P. Liu,[1] R. Bartz,[1] J. K. Zehmer,[1] Y. Ying,[1] M. Zhu,[1] G. Serrero,[2] Y. Zhao,[3] R. G. W. Anderson[1]; [1]Cell Biology, UT Southwestern Medical Center, Dallas, TX, [2]A&G Pharmaceutical Inc, Columbia, MD, [3]Biochemistry, UT Southwestern Medical Center, Dallas, TX

Recent proteomic and immunoblotting analysis of isolated lipid droplets suggests they are specialized organelles involved in membrane traffic. For this reason we have introduced the term adiposome to denote organelle status. Proteins that appear to be highly enriched in adiposomes are the Rab GTPases. Over 17 different Rabs have been identified that are enriched in this compartment. Here we present three lines of evidence that GTPases involved in regulating membrane traffic are functionally associated with adiposomes. First, we assayed for the ability of Rab GDI to remove Rabs from isolated adiposomes. Using immunoblotting we detected the removal of four different Rabs by GDI in the presence of GDPβs but not GTPγs. Next we used the GDI treated adiposomes to see if Rabs could be recruited from cytosol. Multiple Rabs including Rab5 were recruited and recruitment was dependent on both GTP and the concentration of cytosol. Recruitment appeared to be saturable and individual Rabs did not compete with each other, suggesting the presence of Rab-specific binding sites on adiposomes. The Rab5 effector EEA1 was also recruited to adiposomes in the presence of GTP but not other nucleotides. We also found that multiple Arf GTPases were recruited to adiposomes in response to GTP, including Arf1. Interestingly, coordinate with Arf1 recruitment, the coatomers βCOP and εCOP appeared, suggesting membrane budding activity is associated with adiposomes. These results support our hypothesis that adiposomes are organelles involved in membrane lipid traffic.

601

**Lipid Body (Droplet) Metabolism is Controlled by Patatin-like Phospholipase Domain Containing Protein 5 (PNPLA5)**

E. B. Goldberg, W. J. Brown; Molecular Biology & Genetics, Cornell University, Ithaca, NY

Lipid bodies (lipid droplets, adiposomes) are highly conserved storage organelles for neutral lipids and cholesterol esters. Recently, a new family of cytoplasmic lipases named Patatin-like Phospholipase Domain Containing proteins (PNPLA1-5) has been discovered to play a key role in the hydrolysis of stored triglycerides and in the regulation of lipid body metabolism. We report here that PNPLA5, a previously uncharacterized member of this family, also plays an instrumental role in lipid body maintenance. In transfected HeLa cells grown under normal culture conditions, GFP-PNPLA5 was found diffuse in the cytoplasm and partially associated with lipid bodies. However, following induction of lipid body formation with excess oleic acid, GFP-PNPLA5 was located primarily on lipid bodies. Importantly, following lipid loading, over-expression of GFP-PNPLA5

visibly reduced the size of induced lipid bodies. In addition, when cells were starved of lipids to deplete the lipid body pool, GFP-PNPLA5 was found completely cytoplasmic. Finally, following lipid loading, GFP-PNPLA5 was found to co-localize with another family member, adiposome triglyceride lipase (ATGL) (a.k.a. PNPLA2), although ATGL was more punctate and less uniform in distribution on lipid bodies than GFP-PNPLA5. Our data reveals that PNPLA5 localizes to the outside of lipid bodies, much like the pattern previously reported of other PNPLA family members. PNPLA5 plays an important role in the degradation of lipid bodies, most likely by hydrolysis of triglycerides.

602

**Regulation of Lipid Accumulation by ADP Ribosylation**

R. Bartz,[1] J. Seemann,[1] J. K. Zehmer,[1] G. Serrero,[2] K. Chapman,[3] R. G. W. Anderson,[1] P. Liu[1]; [1]Cell Biology, UT Southwestern Medical Center at Dallas, Dallas, TX, [2]A&G Pharmaceutical Inc., Columbia, MD, [3]Department of Biological Sciences, University of North Texas, Denton, TX

Lipid droplets, or adiposomes, are intracellular organelles that are composed of a neutral lipid core (e.g. triacylglycerol and cholesterol esters) surrounded by phospholipids and proteins. While the biogenesis of these organelles remains elusive, the function of lipid droplets in fat storage is clearly established. Here, we report that fat storage in these organelles is regulated by ADP ribosylation. Previous work has shown that caveolin-1 (Cav-1) is associated with adiposomes and that brefeldin A (BFA) causes the amount of Cav-1 in this compartment to increase. We find that short-term (30 min) exposure of cells to BFA causes a marked increase in the amount of Cav-1 in adiposomes, which is not blocked by protein synthesis inhibitors. The BFA stimulated enrichment of Cav-1 presumably is caused by the migration of Cav-1 from other sites in the cell. Unexpectedly, if we incubate cells longer (5-12 hr) in the presence of BFA, lipid droplets completely disappear from the cell. This effect of BFA is dependent on protein synthesis, which suggests that BFA is affecting gene expression. We determined that BFA is enhancing the secretion of fatty acids into the media. Using multiple approaches we determined that the affects of BFA are not due either to a stimulation of the unfolded protein response or to disruption of the Golgi apparatus. BFA is known to stimulate the mono-ribosylation of both the transcriptional regulator C-terminal binding protein (CTBP) and glyceraldehyde-3-phosphate dehydrogenase. Importantly we found that BFA stimulated lipid loss is blocked by two different inhibitors of protein ribosylation, nicotinamide and 3-aminobenzamide. We speculate that lipid storage in many cell types is controlled through mono-ADP ribosylation of proteins involved in transcriptional regulation.

603

**Proteomic Analysis of *Drosophila* Embryonic Lipid Droplets: Storage Depots for Maternally Provided Proteins**

S. Cermelli,[1] Y. Guo,[2] M. A. Welte,[2] S. P. Gross[1]; [1]Dev. and Cell Bio, University of California, Irvine, Irvine, CA, [2]Department of Biochemistry, Brandeis University, Waltham, MD

Lipid droplets are ubiquitous energy-storage organelles of eukaryotes. Despite characterized central roles for cholesterol homeostasis and lipid metabolism, their function is poorly understood. To determine the protein complement of lipid droplets from developing Drosophila embryos, we purified lipid droplets by floatation to near homogeneity, as judged by the absence of markers for other organelles, and analyzed the associated proteins by 2D gel electrophoresis and capillary LC-MS/MS. This sensitive approach allowed us to identify many more proteins (~250) than previously published for lipid droplets from other systems. Important functional groups include known droplet-associated proteins, enzymes involved in fatty acid metabolism, signaling molecules, proteins involved in membrane trafficking, and cytoskeleton associated proteins. In particular, we find homologues of most of the proteins previously identified to be present on mammalian lipid droplets. It should therefore be possible to use Drosophila embryonic lipid droplets as a model for studies of lipid metabolism and related diseases in humans. The list of droplet-associated proteins is surprisingly diverse. Among the unexpected candidates are histone proteins. Using a combination of biochemistry, genetics, real-time imaging and cell biology, we confirm that both endogenous histones and a Histone-GFP fusion protein are present in large quantities on lipid droplets of early embryos, but not of cultured cells. Quantitation of GFP signal and transplantation experiments suggest that histones are transferred from droplets to nuclei as development proceeds. As histone association with droplets starts during oogenesis, droplets may sequester maternally provided histones until they are needed. More generally, we propose that lipid droplets can serve as transient storage depots for proteins that lack appropriate binding partners in the cell. We propose that such sequestration is not only important for early development, but provides a general strategy for handling excess proteins.

604

**The Crystal Structure of Yeast Protein Disulfide Isomerase Reveals Asymmetry Between Its Active Sites**

G. Tian,[1] S. Xiang,[2] W. J. Lennarz,[1] H. Schindelin[2]; [1]Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY, [2]Department of Biochemistry and Cell Biology and Center for Structural Biology, Stony Brook University, Stony Brook, NY

Protein disulfide isomerase is an enzyme that catalyzes the formation and rearrangement of disulfide bonds in newly synthesized proteins. It contains four domains, two catalytically inactive thioredoxin domains inserted between two active thioredoxin domains, and an acidic C-terminal tail. We have determined the crystal structure of yeast PDI at a resolution of 2.4 Å and found that the four thioredoxin domains are arranged in the shape of a twisted “U” with the active sites facing each other across the long sides of the “U”. The inside surface of the “U” is enriched in hydrophobic residues, thereby facilitating interactions with misfolded proteins. The overall domain arrangement, active site location and surface features strikingly resemble the *Escherichia coli* disulfide isomerase DsbC. Based on the structure, site-directed mutagenesis and domain deletion studies were carried out. Biochemical studies demonstrate that all domains of PDI, including the C-terminal tail, are required for full catalytic activity and the two active sites of yeast PDI were revealed to be asymmetric in the enzyme’s catalytic functions. (Supported by NIH grant GM33184 to WJL and DK54835 to H.S.)

605

**Quality Control of a Bacterial Periplasmic BraC Protein in Mammalian Cells**

Y. Shono, H. Shinmura, Y. Nagahara, M. Tanaka; Biotechnology, Tokyo Denki University, Saitama, Japan

The folding and assembly of secretory protein is monitored by chaperons such as calnexin and calreticulin throughout their binding abilities to a specific type of the N-liked oligosaccharide in the endoplasmic reticulum (ER). We, then, wondered how a secretory protein having no conventional N-glycosylation site (-NXT/S-) is discriminated in the system of ER quality control. To study the quality control of secretory protein

with no N-glycan, we transiently transfected a plasmid, pSV2secBraC encoding a bacterial LIVAT-binding protein (BraC protein) fused with a secretion signal of MHC class I H2-$K^d$, into COS-7 cells. Immunoprecipitation with anti-BraC antibody and the subsequent western blotting analysis of the transfected cells showed several molecular species of BraC proteins with higher molecular weights other than a matured BraC protein. Addition of either N-glycosidase F or endoglycosidase H to the immuno-beads diminished the molecular species with higher molecular weight, so a part of the heterogeneity is due to the modification with an N-linked glycan curiously attached to the protein without any N-glycosylation sites. To test the sequence requirements of the modification, we next transfected some derivatives of the pSV2secBraC into COS-7 cells. The modification did not occur when the addition of other amino acid residues or peptides to the C-terminus of BraC protein. Also the removal of the C-terminal lysine of BraC protein brought the modification of BraC protein to a halt. Moreover, the modification occurs when secretion signal was changed to other secretory signals instead of MHC class I H2-$K^d$. This phenomenon was obtained from human hepatoma cells (HuH-7) transiently transfected with pSV2secBraC. Consequently, this unusual modification of N-linked glycan must occur in wide variety of cells including human cells and have an important role in quality control of proteins.

606

**The Quality Control of Human Blood Coagulation Factor X Without N-glycan**

H. Kawashima, Y. Nagahara, M. Tanaka; Tokyo Denki University, Hiki-Gun, Japan

The quality control of secretory glycoprotein is involved in calnexin and/or calreticulin binding cycle in the endoplasmic reticulum. However, the molecular mechanism of quality control of non-glycosylated protein is obscure. To study the mechanism, we constructed plasmids expressing human blood coagulation factor X (FX) which has no N-glycosylation sites. We obtained three plasmids that encode FX mutants with single N-glycosylation site, T223A or T233A, and both of them, T223A and T233A by site-directed mutagenesis. These plasmids were transiently transfected into COS-7 cells, and mutated FX was visualized with immunoblotting. FX mutants were detected in both the cells and the medium. The two mutants, FX(T223A) and FX(T233A) in the cells showed their apparent molecular weight 4kDa lower than that of FX precursor, while molecular weight of the mutant, FX (T223A/T233A) showed further 4kDa lower than that of two mutants. Since these differences among FX and the mutants in the cells were diminished with N-glycosidase F, these differences were due to the N-glycan moieties. FX mutants in the medium seemed to be lower size than intracellular FX mutants. Differences of apparent molecular weights between wild type and the mutants of FX in the medium were diminished with N-glycosidase F, but they had no susceptibilities with Endoglycosidase H. The FX mutants would be normally transported from ER through Golgi network into medium. Similar results obtained from the human hepatoma cells, HuH-7, transiently transfected with FX (T223A/T233A). Furthermore, we established stable transfectants of BALB/c 3T3 cells expressing and secreting the FX (T223A/T233A) to the medium. These results suggest that the FX does not always require its N-glycan for secreting from the cell. An unidentified molecular system may also participant to the quality control of protein.

607

**The Skeletal Muscle Isoform of Calsequestrin is Retained within Streaming Cisternae of Endoplasmic Reticulum Bound to a Subset of Microtubules**

T. D. Houle, M. L. Ram, P. Sateesh, S. E. Cala; Physiology, Wayne State University, Detroit, MI

Cardiac (CASQ2) and skeletal muscle calsequestrin (CASQ1) are abundant lumenal ER/SR proteins in muscle cells. Each CSQ isoform co-traffics with other protein components of the respective Ca2+-release complexes in heart and muscle, although the trafficking steps leading to its eventual concentration in terminal cisternae remain unknown. This trafficking is important to understand because it is so completely altered in animal models of heart failure, and might have a role in disease progression. To study the early steps in CSQ trafficking through the secretory pathway, we overexpressed the two isoforms of CSQ in several nonmuscle cell lines. As we recently reported, cardiac CSQ is retained in ER compartments in all nonmuscle cells. Confocal imaging of CSQ-containing compartments showed a co-localization with GRP94 and calnexin. CSQ was usually restricted to more perinuclear cisternae, and often appeared as a few distinct polygons. In dramatic contrast, skeletal muscle CSQ displayed a peculiar streaming pattern of ER that appeared to emanate from ER and extend to the microtubule-organizing center (MTOC), and was disrupted by the microtubule depolymerizing agent nocadazole. DsRed fusion proteins generated intracellular patterns for CSQ that were somewhat distinct, yet the streaming ER patterns remained vivid. Live cell imaging revealed a highly dynamic streaming ER, with CSQ appearing to move retrogradely from the MTOC and distal ER towards the cell periphery. Interestingly, when CSQ2 was co-overexpressed with the cardiac CSQ cDNA, streaming ER disappeared and both proteins co-localized in a typical cardiac pattern. Our data indicate that unique isoform-specific sequences in CSQ can retain the protein in ER, or permit trafficking beyond ER exit sites. As lumenal ER cargo, CSQ isoforms may contain isoform-specific binding sites to permit interaction with microtubule motor complexes.

608

**Polymyxin B Decreases the Permeability of the Rough Endoplasmic Reticulum to Small Molecules**

W. F. Wonderlin; Biochemistry & Molecular Pharmacology, West Virginia University, Morgantown, WV

We have previously proposed that the release of a nascent chain from a translationally-active, ribosome-bound translocon (RBT) creates an open pore through which small molecules can cross the membrane of the rough endoplasmic reticulum (RER) (JBC, 276:22655; JBC, 278:4397), but a lack of pharmacological tools has limited our study of this pathway. We have continued our study of the permeability of the RER in CHO-K1 cells to 4-methylumbelliferyl-α-D-glucopyranoside (4MG), whose entry can be detected when it is hydrolyzed by lumenal α-glucosidase II. We report here that the increased entry of 4MG produced when nascent chains are released by puromycin (PUR) can be blocked by Polymyxin B (PMB), a cyclic decapeptide with five positively-charged residues and a 6-carbon tail. The effect of PMB on the PUR-stimulated entry of 4MG was biphasic, with modest stimulation at low concentrations and complete inhibition at higher concentrations ($K_i$ of 20-35 μg/ml, ~20 μM). PMB also produced a biphasic stimulation and inhibition of the basal (*i.e.*, PUR independent) entry of 4MG. PMB-nonapeptide, an analog of PMB in which the lipid tail and one charged residue have been removed, produced similar biphasic effects, but with a 2-fold decrease in potency. Although PMB completely blocked the PUR-stimulated entry of 4MG, it reduced the basal permeability by only 10-20%, considerably less than the >80% reduction observed when high salt was used to strip translationally-inactive ribosomes from the RER. We surmise that the partial block of the salt-sensitive entry of 4MG reflects the entry of 4MG via multiple states of the open RBT, or perhaps additional RBT-independent pathways, of which only one is

inhibited by PMB. PMB should be a useful tool for dissecting the pathways for small molecules to cross the membrane of the RER.

609

**Ubiquitin Modification of Serum and Glucocorticoid-induced Protein Kinase (SGK-1) is Associated with its Subcellular Localization**

D. R. Brickley, J. Nicolarsen, L. Belova, A. M. Bogusz, S. D. Conzen; Department of Medicine, University of Chicago, Chicago, IL

SGK-1 is a member of the AGC family of serine/threonine kinases and shares close sequence homology to AKT-1/PKB. Unlike AKT-1, SGK-1 mRNA expression is induced by glucocorticoids, aldosterone, serum, and in response to various environmental stressors. In addition, SGK-1 is rapidly degraded by the ubiquitin-proteasome pathway. In breast epithelial cells, we have previously observed that ubiquitin-modified SGK-1 is found exclusively in a membrane-associated cellular fraction. By examining both immunofluorescent detection of endogenous SGK-1 protein and SGK-1 GFP fusion proteins, we show here that wild type (WT) SGK-1 localizes primarily to the endoplasmic reticulum (ER) and mitochondria; however when aa10-24 are deleted (Dl10-24-SGK-1), SGK-1 localization is disrupted, and the protein is distributed homogenously throughout the cell. Furthermore, Dl10-24-SGK-1 is neither ubiquitinated nor degraded as efficiently as WT SGK-1. Interestingly, deletion of aa15-18 disrupts SGK-1's ER targeting and causes almost exclusive mitochondrial targeting. This suggests that within aa15-18 there may exist an ER localization/retention sequence which when deleted results in a default localization to the mitochondria. Thus far, our mutational analysis has demonstrated that SGK-1's ubiquitin modification and its localization to the ER/mitochondria are correlated, suggesting that SGK-1 localization may be required for interaction with its E3 ligase. We have recently found that the chaperone-dependent E3 ligase, CHIP, is required for SGK-1's rapid protein turnover. Indeed, both SGK-1 and CHIP appear to co-localize at or near the ER. Studies are ongoing to determine whether apoptotic stress induces differential subcellular localization and/or ubiquitin-mediated degradation of endogenous SGK-1.

610

**Stable Ribosome Binding to the Endoplasmic Reticulum Enables Compartment-Specific Regulation of mRNA Translation**

S. B. Stephens,[1] R. D. Dodd,[1] J. W. Brewer,[2] P. J. Lager,[3] J. D. Keene,[3] C. V. Nicchitta[1]; [1]Department of Cell Biology, Duke University Medical Center, Durham, NC, [2]Department of Microbiology and Immunology, Loyola University, Chicago, IL, [3]Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC

mRNAs are distributed between the cytosol and endoplasmic reticulum (ER) compartments of eukaryotic cells by a process of positive selection; mRNAs encoding signal sequences are co-translationally directed to the endoplasmic reticulum (ER) via the signal recognition particle pathway. Following the termination of translation, ER-bound ribosomes are thought to dissociate from the ER to rejoin a common cytosolic pool, thereby completing a translation-driven cycle of RNA partitioning. At present little is known regarding the physiological basis for termination-coupled ribosome release. We examined ribosome and mRNA partitioning during the unfolded protein response, a stress response pathway that suppresses initiation and thereby synchronizes translational termination. We report that physiological induction of termination results in the accumulation, rather than the expected release, of ER-associated ribosomes. Under these conditions, cytosolic translation is suppressed and mRNA/ribosome complexes undergo efficient disassembly. In contrast, translation on the ER is sustained, with mRNAs being present in small polyribosome complexes. Significantly, ER-bound ribosomes were found to participate in the translation of mRNAs encoding soluble as well as secretory proteins. These studies demonstrate that ribosome exchange on the ER is independent of termination and identify new and unexpected roles for ER-bound ribosomes in the translational regulation of cellular protein synthesis.

611

**Vaccinia Virus Replication and Assembly: Manipulation and Utilization of Intracellular Membranes**

P. Traktman, J. P. Mercer, B. Unger; Microbiol. & Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI

Vaccinia virus replicates solely within the host cell cytoplasm. Among the ~200 viral proteins are those mediating genome replication and virion morphogenesis. These processes are regulated by viral protein kinases and involve the remodeling of membranes of the secretory pathway. We have found by immunoelectron microscopy, using antisera directed against cellular PDI, the viral SSB, and BrdU, that viral DNA replication occurs within cytoplasmic foci surrounded by ER. We will present a time course of the formation and enlargement of these sites and address the interactions between ER and viral replication proteins. The virion membrane has been proposed to assemble *de novo* or to derive from cellular ERGIC; recent reports dispute any role for COP II or COP I machinery. We will present new insights into the biogenesis of the virus membrane. (I) Inhibition of the viral F10 kinase prevents membrane biogenesis; F10 binds to PIPs and associates with membranes. Morphogenesis resumes synchronously when *ts*F10-infected cultures are shifted to the permissive temperature. Inclusion of H89, an inhibitor of ER exit site formation, or cerulenin, an inhibitor of *de novo* lipid synthesis, block the restoration of virion production. (II) Phosphoprotein profiling of cellular signaling molecules indicates that the phosphorylation status of several associated with membrane trafficking changes upon infection. (III) Iodixanol gradient fractionation has allowed us to isolate "viral vesicles" seen when viral proteins required for membrane biogenesis are repressed. These membranes are distinct from the ER, ERGIC or Golgi and contain key proteins destined for encapsidation into the virion membrane. Progress in understanding the origin of these membranes, as well as their lipid and protein content, will be discussed.

612

**Characterizing the Substrate Binding Domain of ERdj3, a Mammalian ER DnaJ Protein**

Y. Jin, L. Hendershot; St.Jude Children's Research Hospital, Memphis, TN

ERdj3 has been identified as a soluble mammalian endoplasmic reticulum (ER) DnaJ family member. We previously demonstrated the existence of a large preassembled ER-localized multi-protein complex including BiP and other chaperones. Binding of the complex to unassembled, incompletely folded immunoglobulin heavy chain is accompanied by ERdj3, which is not part of the preassembled complex. *In vivo* binding assays demonstrated that an ERdj3 mutant, which does not interact with BiP, binds to unfolded substrates even better than wild type ERdj3. This result strongly suggests that ERdj3 binds to substrates directly, which was verified by *in vitro* binding assays. While the interaction of DnaJ proteins with their Hsp70 partner is well characterized, less is understood about the interaction of DnaJs with substrates. In order to begin mapping ERdj3's substrate binding domain, we aligned the amino acid sequence of ERdj3 with Ydj1 and Sis1, two yeast cytosolic DnaJs for which structural data are available. Unlike Sis1 which lacks the Cys-rich domain II that is inserted into domain I of Ydj1, ERdj3 is predicted to possess a domain II,

albeit a smaller and atypical Cys-rich domain as compared to Ydj1. Based on the crystal structures of Ydj1 and Sis1 and the predicted secondary structure of ERdj3, we defined domain boundaries. Deletion mutants were made and in vivo binding assay were performed. Our data indicate that domain II (which is very highly conserved evolutionarily among ERdj3s) plays a critical role in the binding of ERdj3 to substrates and that domain Ia also contributes. Delineation of the ERdj3 substrate binding domain is being confirmed by testing the ability of recombinant ERdj3 mutant proteins to bind and refold non-native proteins and to block the aggregation of denatured proteins.

613

**Exploration of the Functional and Organizational Principles of the Yeast Early Secretory Pathway Through An Epistatic Mini Array Profile (E-MAP)**

M. B. Schuldiner,[1] N. J. Krogan,[2] S. R. Collins,[1] N. J. Thompson,[2] V. Denic,[1] A. Bhamidipathi,[1] T. Punna,[2] J. Ihmels,[1] B. Andrews,[2] C. Boone,[2] J. F. Greenblatt,[2] J. S. Weissman[1]; [1]CMP, UCSF, San Francisco, CA, [2]Banting and Best Department of Medical Research,, University of Toronto, Toronto, ON, Canada

We present a strategy for generating and analyzing comprehensive genetic interaction maps, termed E-MAPs (Epistatic Mini Array Profiles), comprising quantitative measures of aggravating and buffering or suppressing interactions between gene pairs. Crucial to the analysis of E-MAPs is their high-density nature made possible by focusing on largely self-contained gene subsets and including essential genes. To enable genetic analysis of essential proteins we developed a novel technique, termed DAmP (Decreased Abundance by mRNA Perturbation) for rapidly constructing hypomorphic alleles. Described here is the analysis of an E-MAP of genes acting in the early secretory pathway (ESP) of *Sacchromyces cerevisiae*. Hierarchical clustering of our interaction network, together with the application of novel analytical strategies and specific experimental verification revealed or clarified the role of many proteins in the ESP. At a broader level, analysis of the E-MAP delineated pathway organization and components of physical complexes, and illustrated the interconnection between the various secretory processes. Extension of this strategy to other logically connected gene subsets in yeast and higher eukaryotes should provide critical insights into the function and organizational principles of biological systems.

614

**The Cytoplasmic Hsp70-chaperone Machinery Subjects Misfolded and ER Import Incompetent Proteins to Degradation via the Ubiquitin-proteasome System**

S. Park,[1] N. Bolender,[1] J. Takeuchi,[2] P. Coffino,[2] D. H. Wolf[1]; [1]Institut für Biochemie, Universität Stuttgart, Stuttgart, Germany, [2]Dept. of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA

We have shown in yeast that degradation of CPY*, a soluble misfolded protein of the endoplasmic reticulum, requires the trimeric Cdc48-Ufd1-Npl4 complex as well as the UBA-UBL proteins Dsk2p and Rad23p of the cytosol for their degradation via the ubiquitin-proteasome system (Kostova and Wolf (2003) EMBO J 22, 2309-2317; Medicherla et al., (2004) EMBO Rep. 5, 692-697). Here we show that ER import defective CPY* (Δss CPY*), derivatives thereof (e.g., ΔssCPY*-GFP) or ER import incompetent wild type CPY are rapidly degraded via the cytosolic ubiquitin proteasome system. In contrast to the soluble ERAD substrate CPY*, degradation of these proteins is independent of the trimeric Cdc48 complex as well as of Dsk2p and Rad23p. Instead, their degradation requires the cytosolic Hsp70 chaperones of the Ssa type as well as a variety of co-chaperones. Most interestingly, while proteasomal degradation of signal sequence deleted, cytoplasmically localized ΔssCPY*-GFP requires the Ssa1-machinery, degradation of a GFP fusion containing the C-terminal 37 amino acid region of ornithine decarboxylase, a ubiquitin-independent degradation tag (Zhang et al. (2003), EMBO J. 22, 1488-1496), is independent of this machinery.

615

**ERp29 Triggers a Conformational Change in Polyomavirus to Stimulate Membrane Binding**

E. K. Rainey, B. Magnuson; Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI

Membrane penetration of non-enveloped viruses is a poorly understood process. We have investigated early stages of this process by studying the conformational change experienced by polyomavirus (Py) in the lumen of the endoplasmic reticulum (ER), a step that precedes its transport into the cytosol. We show that a PDI-like protein, ERp29, exposes the C-terminal arm of Py's VP1 protein, leading to formation of a hydrophobic particle that binds to a lipid bilayer; this reaction likely mimics initiation of Py penetration across the ER membrane. Expression of a dominant-negative ERp29 decreases Py infection, indicating ERp29 facilitates viral infection. Interestingly, cholera toxin, another toxic agent that crosses the ER membrane into the cytosol, is unfolded by PDI in the ER. Our data thus identify a novel ER factor that mediates membrane penetration of a non-enveloped virus and suggest that PDI family members are generally involved in ER remodeling reactions.

## Epithelia I (616-630)

616

**Polarity Establishment and Orientation during Morphogenesis are Regulated by Two Distinct Molecular Cues**

A. Datta,[1] W. Yu,[1] K. D. Liu,[1] P. R. Brakeman,[1] T. Jou,[2] M. M. P. Zegers,[3] K. E. Mostov[1]; [1]Departments of Anatomy and Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA, [2]Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan Republic of China, [3]Epithelial Pathobiology, Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, OH

Normal epithelial cells must form and maintain a polar architecture for their function *in vivo*. Development of epithelial organs requires both the establishment of polarity as well as the determination of the orientation of polarity. Using MDCK epithelial cysts grown in a 3-dimensional collagen matrix as a model system, we sought to distinguish the pathways that regulate the orientation vs. establishment of polarity. β-1 Integrin mediated downstream signals have been shown to regulate the orientation of polarity in MDCK cysts through a Rac1 dependent pathway (*O'Brien et.al*, Nat. Cell Biol., 2001, 3(9):831, *Yu et.al.* Mol. Biol. Cell 2005, 16(2):433). Here we report that Cdc42 is a key component of the pathway that establishes polarity in 3-dimensions. The loss of Cdc42 function leads to a loss of polarity in a 3-dimensional collagen culture. The loss of Rac1, in contrast, has been shown to lead to an inversion of polarity orientation. The expression of a constitutively activated Cdc42 can bypass the

requirement for Integrin signal for determining the orientation of polarity. However, in contrast to Rac1, the addition of exogenous laminin is not sufficient to rescue the polarity defect due the loss of Cdc42, indicating that Cdc42 acts downstream of Integrin-ECM signals in the polarity pathway. While Rac1 regulates the orientation of polarity, our data indicates that Cdc42 is a part of the cellular machinery that provides the ultimate spatial cue for the establishment of epithelial polarity during morphogenesis.

617

**Apical Targeting of Syntaxin 3 is Essential for Epithelial Cell Polarity**

N. Sharma,[1] S. Low,[1] S. Misra,[2] B. Pallavi,[1] T. Weimbs[1]; [1]Department of Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA, [2]Department of Molecular Cardiology, Cleveland Clinic, Cleveland, OH

In epithelial cells, syntaxin 3 localizes to the apical plasma membrane and is involved in membrane fusion of apical trafficking pathways. Here, we show that syntaxin 3 contains a necessary and sufficient apical targeting signal centered around a conserved FMDE motif. Mutation of any of three critical residues within this motif leads to loss of specific apical targeting. Modeling based on the known structure of syntaxin 1 revealed that these residues are exposed on the surface of a three-helix bundle. Syntaxin 3 targeting does not require binding to Munc18b. Instead, syntaxin 3 recruits Munc18b to the plasma membrane. Expression of mis-localized, mutant syntaxin 3 in MDCK cells leads to basolateral mis-targeting of apical membrane proteins, disruption of tight junction formation, and loss of ability to form an organized polarized epithelium. These results indicate that SNARE proteins contribute to the overall specificity of membrane trafficking in vivo, and that the polarity of syntaxin 3 is essential for epithelial cell polarization.

618

**Muc4-ErbB2 Complex Formation and Signaling in Polarized CACO-2 Epithelial Cells**

K. L. Carraway,[1] V. P. Ramsauer,[1] P. J. I. Salas,[1] C. A. C. Carraway[2]; [1]Cell Biology and Anatomy, University of Miami School of Medicine, Miami, FL, [2]Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL

This study was initiated to determine the mechanisms by which the membrane mucin Muc4 interacts with ErbB2 and regulates ErbB2 signaling in polarized epithelial cells. Muc4 serves as an intramembrane ligand for the receptor tyrosine kinase ErbB2. To examine the stoichiometry and formation of the Muc4-ErbB2 complex, the soluble, extracellular domain forms of Muc4 and ErbB2 were expressed with the Baculovirus Expression Vector System in High Five insect cells, as previously reported for secretion of the complex. The complex was purified by affinity chromatography and immunoprecipitation and biotinylated to determine its stoichiometry, which was 1:1. Using pulse-chase analyses, the time to complex formation was determined to be less than 15 min, compared to 45-60 min for complex secretion. In polarized CACO-2 cells, Muc4 expression causes localization of ErbB2, but not its heterodimerization partner ErbB3, to the apical cell surface, effectively segregating the two receptors. The apically located ErbB2 is phosphorylated on tyrosines 1139 and 1248, as determined by specific anti-phospho-ErbB2 antibodies. The phosphorylated ErbB2 in CACO-2 cells recruits the cytoplasmic adaptor protein Grb2, consistent with previous studies showing phosphotyrosine 1139 to be a Grb2 binding site. To address the issue of downstream signaling from apical ErbB2, we analyzed the three MAPK pathways of mammalian cells, Erk, p38 and JNK. Consistent with the more differentiated, polarized phenotype of the CACO-2 cells, p38 phosphorylation was robustly increased by Muc4 expression, with consequent activation of Akt. In contrast, Erk phosphorylation was weakly increased, and JNK phosphorylation was not changed. The ability of Muc4 to segregate ErbB2 from the other ErbB receptors and to alter downstream signaling cascades in polarized epithelial cells suggests that it has a role in regulating ErbB2 functions in differentiated epithelia.

619

**Lumen Formation and Tubulogenesis by MDCK Cells in Three-Dimensional Cultures Studied with Selective Plane Illumination Microscopy**

F. Pampaloni,[1] E. G. Reynaud,[1] K. Greger,[1] M. Marcello,[2] J. Swoger,[1] E. H. K. Stelzer[1]; [1]Cell Biology and Biophysics Programme, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, [2]German Cancer Research Center, Heidelberg, Germany

It is known that Madin-Darby Canine Kidney cells form apicobasal polarized spherical shells (cysts) when grown in a three-dimensional matrix like Matrigel or collagen. MDCK cells and other 3D cellular model systems are very valuable to investigate the formation and function of glandular epithelia. We applied Selective Plane Illumination Microscopy (SPIM) to study the sequence of spatially and temporally regulated morphogenic events leading to lumen formation and branching tubulogenesis in 3D-cultured MDCK cells. SPIM is a new high-resolution fluorescence microscopy technique that has been developed for the modern life sciences. For the emerging field of 3D cell biology SPIM is an ideal instrument. It enables researchers to image a whole population of cells in their 3D context,to visualize their morphology in a matrix and to track cells through the context of surrounding tissue. In SPIM, detection of fluorescence light perpendicularly to the illumination axis (theta principle of detection) is combined with selective excitation of fluorophores within an entire plane by a focused laser light sheet. Therefore, selective plane illumination provides optical sectioning directly. Bleaching outside the volume of interest is avoided. Moreover, CCD camera-based wide-field detection provides high recording speed.

620

**Transglutaminase is Involved in UVB-induced NF-κB Activation in Corneal Epithelial Cells**

L. Tong,[1,2,3] R. M. Corrales,[1] L. Z. Chen,[1] C. S. de-Paiva,[1] D. Q. Li,[1] S. C. Pflugfelder[1]; [1]Ocular Surface Center, Cullen Eye Institute, Baylor College of Medicine, Houston, TX, [2]Singapore National Eye Center, Singapore, Singapore, [3]Singapore Eye Research Institute, Singapore, Singapore

Anti-transglutaminase(TG) strategies reduce inflammation in allergic conjunctivitis; however, the role of TG in the pro-inflammatory pathways in ocular surface epithelial cells has not been investigated. A human cornea epithelial cell line(T-HCEC) and primary human corneal epithelial cells(PCE) cultured from limbal explants were used. Cells were stimulated with a single dose of UVB at 20 $mJ/cm^2$ or tumour necrosis factor (TNF-α). RNA levels were assessed by RT-PCR and protein expression by immuno-cytochemistry and Western blotting. Culture media cytokine concentration was assayed with a bead-based sandwich immuno-fluorescence assay. TG activity was assessed by fluorescein-cadaverine uptake. UVB induced the clustering of TNF-α Receptor-I, which was inhibited by monodansyl-cadaverine(MDC), a TG inhibitor. UVB also stimulated

phosphorylation of JNK, as well as increased TG activity. Incubation with SP600125, a JNK inhibitor, reduced UVB-induced TG activity. The expression of TG-1 and TG-2 proteins was stimulated by UVB, but suppressed by MDC or SP600125. Similarly, TG-1 RNA expression was also stimulated by UVB and suppressed by MDC. UVB induced the translocation of p65 into nuclei of cells, activating the NFκB pathway: a similar effect to TNF-α stimulated cells. Incubation with MDC reduced this translocation, an effect similar to that of SN-50, a peptide inhibitor of p65 translocation. MDC also suppressed the TNF-α stimulated nuclear translocation of p65. TNF-α and IL-8 secretion into culture media were increased after UVB stimulation. Incubation with MDC reduced the concentration of TNF-α, but not of IL-8 after UVB. In cornea epithelial cells, TG can mediate activation of the stress induced NFκB pathway, secreting pro-inflammatory cytokines in a cytokine-selective fashion. UVB signaling involves TG dependent clustering of cell surface receptors. JNK may be an upstream signal for TG expression and activation. This pathway offers potential targets for treatment of ocular surface diseases.

621

**Zinc Transport in Proliferating and Differentiated Trophoblasts (BeWo) is Regulated Differently by Progesterone and is Associated with Changes in Zip4 and ZnT1 Levels**

X. Du, B. Lonnerdal, S. L. Kelleher; Nutrition, University of California Davis, Davis, CA

Zinc (Zn) is essential for normal fetal growth and development. During implantation, proliferating cytotrophoblasts invade the endothelium, and then differentiate into syncytiotrophoblasts transporting nutrients from mother to fetus. These distinct functions suggest unique Zn requirements. We hypothesize that trophoblast Zn transport is regulated by progesterone (PG), a steroidal hormone critical for trophoblast implantation and function, mediated via Zip4 (Zn uptake) and ZnT1 (Zn efflux). BeWo cells were grown in bicameral chambers or on glass coverslips and differentiated with forskolin (100μM) for 72 h. Cells were treated with PG (1μM) for 1 h, Zn transport was measured, Zip4 and ZnT1 protein abundance was determined by Western blot and localization demonstrated by confocal microscopy. While proliferating and differentiated trophoblasts transport similar amounts of Zn, proliferating cells retain more cellular Zn and differentiated cells efflux more Zn across the basal membrane; however, Zip4 (localized at the plasma membrane) and ZnT1 (localized to intracellular vesicles) abundance was similar. Progesterone reduced total Zn transport and basolateral Zn efflux and increased cellular Zn retention in proliferating cells which was associated with lower ZnT1 and higher Zip4 abundance. Progesterone had no effect on total Zn transport in differentiated cells; however, basolateral Zn efflux was decreased and cellular Zn retention was increased but both Zip4 and ZnT1 abundance were decreased. Our results indicate that proliferating trophoblasts preferentially retain Zn for cellular use while differentiated trophoblasts enhance Zn efflux to the fetus. While PG is essential for the establishment and maintenance of pregnancy, elevated PG during early pregnancy may impair cellular Zn uptake, thus interfering with invasion and during late pregnancy may reduce Zn transfer to the developing fetus through post-transcriptional changes in specific Zn transporters.

622

**Striking Differences in Activities of Antioxidant and Prooxidant Enzymes in the Normal Corneal Epithelium of Various Mammalia**

J. Cejkova,[1] J. Platenik,[2] M. Vejrazka,[2] C. Cejka,[1] S. Stipek,[2] T. Ardan,[1] J. Kovaceva,[1] A. Midelfart[3]; [1]Eye Histochemistry and Pharmacology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic, [2]First Institute of Medical Chemistry and Biochemistry, Charles University, Prague, Czech Republic, [3]Department of Ophthalmology, Faculty of Medicine, Trondheim, Norway

Under normal conditions antioxidants are balanced in the corneal epithelium with reactive oxygen species generation without any toxic effects. The danger from oxidative stress appears when the natural antioxidants are overwhelmed leading to antioxidant/prooxidant imbalance. The aim of the present investigation is to examine activities of enzymes participating in the antioxidant/prooxidant balance in the normal corneal epithelium of various mammalia. The antioxidant enzymes (superoxide dismutase and glutathione peroxidase) and prooxidant enzymes (xanthine oxidoreductase/xanthine oxidase) were investigated biochemically. Results show that the superoxide dismutase activity is high in the rabbit and guinea pig corneal epithelium, whereas in the pig corneal epithelium the activity is low and in the bovine corneal epithelium the activity is nearly absent. In contrast, glutathione peroxidase activity is high in the bovine, pig and rabbit corneal epithelium, whereas in the guinea pig corneal epithelium the activity is low. Prooxidant enzymes reveal high xanthine oxidoreductase/xanthine oxidase activity in the rabbit corneal epithelium and much lower activities in the guinea pig and bovine corneal epithelium. In the pig corneal epithelium the activity is absent. In conclusion, results demonstrate substantial variations in enzyme activities. Further studies are necessary to elucidate whether differences in activities might influence the individual susceptibility to oxidative stress-mediated ocular toxicity. At present our study may offer useful information regarding the activities of antioxidant and prooxidant enzymes in various animal species when corneal epithelial cells are choosing for ocular research.

623

**Identification and Characterization of a Novel Lysophosphatidylcholine Acyltransferase in Alveolar Type II Cells**

X. Chen,[1] B. A. Hyatt,[2] J. M. Shannon,[2] R. J. Mason[1]; [1]Medicine, National Jewish Medical and Research Center, Denver, CO, [2]Pulmonary Biology, Cincinnati Children's Hospital, Cincinnati, OH

Pulmonary surfactant is a complex of lipids and proteins produced in alveolar type II cells. Surfactant provides the low surface tension at the air-liquid interface and prevents alveolar collapse during expiration. Pulmonary surfactant is composed predominantly of phospholipids (~ 85%). Lysophosphatidylcholine (lysoPC) acyltransferase is thought to play a critical role in surfactant dipalmitoylphosphatidylcholine (DPPC) synthesis. However, the acyltransferase involved in DPPC synthesis has not yet been identified. Methods: A rat cDNA clone (R1) was identified in the NCBI data base by its sequence homology to a murine putative acyltransferase that is specifically expressed in the developing lung. The full length R1 gene was amplified by PCR from cDNA of rat alveolar type II cells, resulting in a 1.6 kb product. The R1 gene was subcloned into a mammalian expression vector and transfected into 293 cells and COS-7 cells. The recombinant R1 protein was assayed for lysophospholipid acyltransferase activity by measuring the incorporation of radiolabeled acyl moieties of acyl-CoA ( acyl donors) into phospholipids. Results: The R1 gene predicts a 534 amino acid protein of ~ 59 kDa, which contains a transmembrane domain and an acyltransferase (PlsC) domain. The R1 acyltransferase expressed in 293 cells and COS-7 cells increases lysoPC acyltransferase activity. The recombinant enzyme shows a clear preference for palmitoyl-CoA as the acyl donor. Furthermore, keratinocyte growth factor ( KGF) increases the mRNA expression of R1 acyltransferase 8 fold in rat type II cells, and the expression of R1 decreases markedly when type II cells were cultured on plastic to become type I-like cells. Conclusion: The R1 acyltransferase likely plays an important role in biosynthesis of surfactant phospholipids. The elucidation of R1 gene's regulation will increase our

understanding of the control of surfactant lipid synthesis.

624

**Stretch-Induced Exocytosis of Discoidal Vesicles in Bladder Umbrella Cells is Modulated by Tyrosine Kinase Activity**

E. M. Balestreire, G. Apodaca; Department of Medicine, Renal-Electrolyte Division, University of Pittsburgh, Pittsburgh, PA

The mucosal surface of the bladder forms an impermeable barrier that must accommodate changes in urine volume as the bladder fills and empties. At the cellular level, this is accomplished by the dynamic addition and removal of membrane from the apical surface of the umbrella cell layer. An important stimulus for membrane turnover in umbrella cells is bladder filling; however, the mechanotransduction pathways that lead to changes in exocytosis and endocytosis are not known. Tyrosine phosphorylation plays an important role in transducing mechanical stimuli into downstream cellular events in other cell types including vascular endothelial cells, keratinocytes, kidney proximal tubule cells, and osteoblasts. This study examines the hypothesis that tyrosine kinases are an integral part of the mechanotransduction pathway that leads to exocytosis of discoidal vesicles in the filling bladder. A modified Ussing chamber was used to monitor bladder uroepithelium capacitance, a measure of surface area, upon stretch stimulus. In control bladder tissue, filling induced a 50% increase in surface area over 5 hours. Addition of the broad-spectrum tyrosine kinase inhibitor genistein significantly reduced this response to ~25% over 5 hours. The epidermal growth factor receptor (EGFR) inhibitor AG-1478 also attenuated filling-induced increases in capacitance. Specific inhibitors of tyrosine kinases PDGFR, Src, and Jak2, which are known to be important for mechanotransduction in other cell types, caused no inhibition of filling-induced exocytosis. These studies suggest that tyrosine kinases, particularly EGFR, may be important players in the mechanotransduction pathway found in bladder epithelial cells. The exploration of tyrosine kinase activity in the stretch-induced response of bladder tissue will further our understanding of cellular responses to mechanical stimuli, while providing insights into the normal physiology of bladder accommodation of urine.

625

**BMP Signaling is Essential for the Proper Terminal Differentiation of Intestinal Secretory Lineage in Mice**

B. A. Auclair,[1] Y. Benoit,[1] Y. Mishina,[2] D. L. Gumucio,[3] N. Perreault[1]; [1]Anatomy and Cell Biology, University of Sherbrooke, Fleurimont, PQ, Canada, [2]Environmental Disease & Medicine Program, National Heart Lung and Blood institute, Research Triangle Park, NC, [3]Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

**BACKGROUND**: Bone morphogenetic proteins (BMPs) are morphogenes known to regulate numerous cell processes in various tissues. In the intestine, the BMPs are primary produced by the mesenchymal compartment. These factors can activate their signaling pathways following a paracrine or autocrine route. Recent publications have shown links between BMPs signaling and colorectal carcinogenesis. However, the potential effects of this cascade on other important cell functions in the gastrointestinal tract have not yet been explored. **AIM:** To investigate the paracrine role of BMPs signaling during intestinal epithelial cell fate. **METHODS AND RESULTS**: With the use of the Cre/loxP system, we generated a mouse with BMPR1A receptor exclusively deleted in the gut epithelium by crossing floxed *BMPR1A* mice with the Villin-Cre line. Histology analysis showed that the intestine of *Villin-Cre/BMPR1A*$^{loxP/loxP}$ mice displayed abnormal epithelial morphology with elongated villi and multiplication of the crypt units. BrdU incorporation in adult mice revealed an increased number of proliferating cells in mutant mice. The proliferating cells were not confined to the bottom of the crypt but were spread throughout the crypt. Chromagranin A immunostaining showed a statistically significant increase of the number of enteroendocrine cells in the in *Villin-Cre/BMPR1A*$^{loxP/loxP}$ mice. The number of goblet and Paneth cells was not significantly changed in the mutant animals. However, goblet cells were consistently smaller in the mutant mice as seen in Alcian blue staining. Electron microscopy studies confirmed the reduction in size of the goblet cells. Moreover, immunostaining with an anti-lysosyme antibody suggested a reduction in Paneth cell secretory granule content in *Cre-Villin-Cre/BMPR1A*$^{loxP/loxP}$ mice. **CONCLUSION:** Altogether, these results strongly suggest that paracrine BMPs signaling might play an important role in the terminal differentiation of intestinal secretory cell type.

626

**New Molecular Markers of Human Skin Ageing**

M. Dumas, M. Bonnet Duquennoy, C. Heusele, E. Noblesse, R. Kurfurst, S. Schnebert, F. R. Bonte; LVMH RECHERCHE, Saint jean de braye, France

Skin ageing is mainly due to ultraviolet radiation and oxidative stress leading to modification of its biophysical properties. Studying photoexposed and photoprotected skin biopsies from young and aged women, it has been found that a specific zone, composed of the basal layers of the epidermis, the dermal epidermal junction and the superficial dermis, is the major target of ageing and reactive oxygen species. We showed that this zone named aging responsible interface is characterized by significant variations at a transcriptional and /or protein levels of integrin B1, B4, cytokeratin1, hyaluronic acid, aquaporin 3, tyrosinase, TRP1, p63, p73, type I, III, IV and VII collagens, type 1 laminin, fibrillin, elastic fibers, MMP1, MMP9, TIMP1, PDGF, Chondroitine-4 sulfate and hyaluronate. Skin ageing being mainly characterized by a thinning of the epidermis and loss of its defense and biomechanical properties we decided to focus on epidermal homeostasis and protective mechanisms. Using skin-dedicated cDNA microarray on human keratinocytes (oligosorbent array technique) we found that 24 hours H2O2 treatment downregulates p63, p73 transcription factors known to be involved in the control of the cell cycle regulation and turnover of the epidermis. A natural mixture containing labdane diterpenoids has been found to significantly increase p 63, p73 cell cycle regulators and also the expression of protective thioredoxin genes.

627

**Overexpression of CRABPI in the Epithelial Suprabasal Layer Affects the Proliferation of Epithelial Basal Layer Cells in Retinoic Acid Topically Treated Mouse Skin**

X. Tang; Pharmacology, Medical College of Cornell Univ., New York, NY

CRABPI is a cellular retinoic acid binding protein, and its effects on the physiological functions of retinoic acid are not fully understood. We investigated whether overexpressed CRABPI could influence the actions of retinoic acid in transgenic animals. We specifically targeted CRABPI to the suprabasal layer of the mouse epithelium through the use of the keratin 10 (K10) promoter. CRABPI mRNA was highly expressed in the skin of one week old transgene positive mice as compared to transgene negative mice of the same age. A much higher level of CRABPI protein was

detected in the epidermis of adult, transgene positive mice than in transgene negative mice of the same age. Overexpression of CRABPI did not cause significant morphological abnormalities in adult mouse skin. However, ectopically overexpressed CRABPI in the suprabasal layer enhanced the thickening of the epidermis induced by topical retinoic acid treatment (10 _M, 400 _l for 4 days). It has been reported that the epidermal basal cell proliferation is stimulated by the heparin-binding EGF-like growth factor secreted by retinoic acid activated suprabasal layer cells (Xiao et al., 1999). Our results indicate that a high level of CRABPI in suprabasal layer cells enhances the physiological functions of retinoic acid. Xiao, J. H., Feng, X., Di, W., Peng, Z. H., Li, L. A., Chambon, P. and Voorhees, J. J. (1999). Identification of heparin-binding EGF-like growth factor as a target in intercellular regulation of epidermal basal cell growth by suprabasal retinoic acid receptors. EMBO J 18, 1539-48.

628

**Autocrine Crosstalk in the Response of Human Epithelial Cells to Tumor Necrosis Factor**

K. A. Janes,[1] S. Gaudet,[2] J. G. Albeck,[2] U. B. Nielsen,[3] D. A. Lauffenburger,[4] P. K. Sorger[2]; [1]Department of Cell Biology, Harvard Medical School, Boston, MA, [2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, [3]Merrimack Pharmaceuticals, Cambridge, MA, [4]Biological Engineering Division, Massachusetts Institute of Technology, Cambridge, MA

Tumor necrosis factor (TNF) is a cytokine that induces apoptotic and inflammatory responses, which can be antagonized by growth factors such as EGF and insulin. Here we report a quantitative experimental analysis of crosstalk among TNF, EGF, and insulin receptors in human epithelial cells. Using kinase activity assays, quantitative immunoblotting, and antibody arrays, we construct a cytokine-signaling compendium comprised of ~10,000 protein measurements from HT-29 cells treated with multiple TNF, EGF, and insulin combinations. Mining of the compendium data by classifier-based regression reveals that cells respond to TNF both directly, via activated TNF receptor, and indirectly, via autocrine circuits involving transforming growth factor-α (TGF-α), interleukin-1α (IL-1α), and IL-1 receptor antagonist (IL-1ra). The cascade of TNF-induced autocrine cytokines begins within 10-15 min and evolves over 24 hr to play as important a role as TNF itself in controlling cell signaling and fate. Furthermore, these cytokines show an intricate molecular logic: autocrine IL-1α requires the preceding release of TGF-α, and IL-1α signaling is self-limited by the subsequent release of IL-1ra. The cytokine network exists in colon, breast, and lung epithelial cell lines, suggesting that the autocrine cascade is a general regulatory response of epithelia to TNF. We speculate that time-dependent crosstalk of synergistic and antagonistic autocrine circuits serves to link cellular responses to the local environment during inflammation.

629

**Amino-terminal TIG3 Truncation Mutants Associate with Type 1 Transglutaminase and Induce Keratinocyte Cell Death**

R. Jans, M. T. Sturniolo, R. L. Eckert; Physiology & Biophysics, Case Western Reserve University, Cleveland, OH

Type 1 transglutaminase (TG-1) is a plasma membrane-anchored protein-protein crosslinking enzyme that is responsible for assembly of the cornified envelope during epidermal keratinocyte differentiation. We recently described TIG3, a low molecular weight, membrane-associated protein, as a TG-1 activator protein in keratinocytes. We also demonstrated that the carboxy-terminal membrane-anchoring domain is required for TIG3 associated cell death. In the present study, we examine the role of the TIG3 amino-terminus. Similarly to full-length TIG3(1-164), expression of the N-terminal TIG3 mutant, TIG3(41-164), causes cell death. Interaction of the TIG3(41-164) with TG-1 was identified by co-immunoprecipitation. In addition, immunoblot analysis revealed high molecular weight forms of TIG3(41-164) in TIG3(41-164) expressing keratinocytes. Formation of these structures is inhibited by treatment with monodansylcadaverine, a competitive transglutaminase substrate, suggesting that TIG3(41-164) is a TG-1 substrate. These findings suggest that TIG3(41-164) promotes keratinocyte cell death by interacting with, and activating, TG-1, and suggest that the N-terminal amino-acids of TIG3 are not required for this function. In addition, these findings indicate that TIG3(41-164) can also serve as a TG-1 substrate. Additional studies show that amino-terminal mutants including TIG3(100-164), TIG3(112-164), and TIG3(124-164) also promote cell death. These findings suggest that the TIG3 amino terminus is not required for TIG3 cell death functions.

630

**How Do Keratinocytes Respond to Mechanical Stress ? A Transcriptomal Analysis**

O. Duverger,[1,2] M. Moreau,[1,2] M. Bonnet-Duquennoy,[1] S. Schnebert,[1] C. Nizard[1,2]; [1]R&D LVMH Parfums et Cosmetiques, Saint Jean de Braye, France, [2]INSERM U505, Paris, France

The skin is subjected to various strains. Although several studies have focused on the response of dermal fibroblasts to mechanical stress, the response of keratinocytes to strain is poorly documented. We used the cDNA microarray technology to analyze the response of keratinocytes to stretching. HaCaT cells were cultured on a flexible surface and subjected to cyclic stretching. Their transcriptome was analyzed at different times after the beginning of the treatment. This kinetic analysis allowed to identify early and late mechano-responsive genes, including epidermal markers, adhesion molecules, extracellular matrix components, growth factors, transcription factors, genes involved in signal transduction, in cell metabolism, in inflammatory response, in apoptosis and in cell cycle regulation. These results were confirmed by real-time RT-PCR, for a set of selected genes. Bioinformatics analyses led to the identification of pathways involved in the response of keratinocytes to mechanical stress. In short, keratinocytes subjected to cyclic stretching tend to proliferate, to modify their adhesion properties and to change the composition of their extracellular matrix. Moreover, several corneocyte markers are strongly up-regulated, suggesting an activation of late steps of keratinocyte terminal differentiation, as well as a modification of the biomechanical properties of the stratum corneum. These results will be of great interest for the understanding of skin behaviour in response to strain, in normal and pathological conditions.

## Parasitology (631-654)

631

**Hemoglobin Genotype-dependent Erythrocyte Membrane Potential Correlates to Innate Protection Against Severe Malaria**

F. Tokumasu,[1] J. A. Dvorak[2]; [1]Laboratory of Malaria and Vector Research, National Institutes of Health, Rockville, MD, [2]National Institutes of Health, Rockville, MD

Individuals carrying mutant hemoglobin (Hb) genotypes such as HbC and HbS display innate protection against severe malaria. HbC erythrocytes

age more rapidly than normal erythrocytes with an increase of hemichrome on the cytoplasmic side of the membrane as well as high level of band 3 aggregations. For example, Plasmodium falciparum-infected HbC erythrocytes display larger 'knobs' than parasitized HbA erythrocytes, and decreased endothelial cytoadherence. However, the critical mechanisms responsible for these abnormalities have not been elucidated. One possibility is that physical-chemical features reflected in the lateral membrane organization of lipids and membrane proteins differ between normal and abnormal Hbs. If this premise is correct, the differences should be reflected in the erythrocyte zeta potential as it is an indirect measure of membrane surface potential which can be modulated by variations in membrane proteins and/or lipids as well as exogenous proteins such as immunoglobulins. Homozygous HbA (AA) and HbC (CC) show zeta potentials of -15 and -12 mV, respectively. CC erythrocyte zeta potential distributions also have a longer tails containing higher zeta potential values, implying that various degrees of membrane abnormalities exist in CC erythrocytes. Moreover, aged AA erythrocytes also showed ~1.5 mV higher zeta potential than younger erythrocytes. This implies that CC erythrocytes are prematurely aged but at a higher rate than AA erythrocytes. Furthermore, both P. falciparum-infected AA and CC erythrocytes also show 1-2 mV higher zeta potentials than non-parasitized erythrocytes, indicating that a malaria infection modifies the membrane zeta potential. In contrast, only minor zeta potential differences were found between AA and SS erythrocytes, despite the markedly aberrant shapes induced in SS erythrocytes. Our results demonstrate for the first time that a relationship exists between innate protection against severe malaria and membrane-related physical properties of the erythrocytes.

632

**The Avian Malaria Parasite *Plasmodium gallinaceum* Modifies the Surface Topography of Infected Erythrocytes**

T. Arie,[1] D. W. Dorward,[2] J. A. Dvorak[1]; [1]LMVR, NIAID, Rockville, MD, [2]NIAID/Rocky Mountain Laboratories, Hamilton, MT

In humans, a *Plasmodium falciparum* infection induces knob-like protrusions on the erythrocyte surface. Knobs begin to appear at the late ring stage of the parasite's development. As the intraerythrocytic cycle of malaria progresses the knobs are distributed over the surface of the erythrocyte and the total number of knobs per unit area increases linearly as the parasite matures. These knobs cause the adherence of infected erythrocytes to capillary endothelia as well as other erythrocytes resulting in severe pathologic sequaelae. In contrast to *P. falciparum*, we found by a combination of wide-field fluorescence and atomic force microscopy that the infection of avian erythrocytes by *P. gallinaceum* results in the appearance of furrow-like structures on the infected erythrocyte surface; uninfected erythrocytes do not show this feature. Furthermore, these furrows appear on the surface at almost the same developmental stage of the parasite as do the knob-like structures induced by *P. falciparum*. To further investigate this phenomenon, we utilized scanning and transmission electron microscopy to determine if it was in any way analogous to the knobs formed on infected human erythrocytes by *P. falciparum*. We found that the furrows arise from beneath the erythrocyte membrane, implying that they are probably associated with cytoskeletal components of the erythrocyte membrane. Our data demonstrate that surface modifications resulting from a malaria infection are not restricted to knob-like structures; malaria-induced surface modifications may take other forms. The resolution of this phenomenon could help in our general understanding of the intraerythrocytic cycle of this medically important parasite.

633

**Unexpected Metabolic Pathways in the Apicoplast and Mitochondrion of Toxoplasma gondii**

D. Soldati,[1] J. Limenitakis,[1] L. Kwok,[1] B. Foth,[1] P. Pino,[2] R. Schepers[2]; [1]Microbiology and Molecular Medicine, CMU, University of Geneva, Geneva, Switzerland, [2]Biological Sciences, Imperial College London, London, United Kingdom

The non-photosynthetic plastid-like organelle indispensable for the parasite survival is the site of several anabolic pathways including fatty acid, isoprenoids, iron-sulphur cluster and some steps of the heme biosynthesis. Most enzymes that are active inside the apicoplast are encoded by the nuclear genome and targeted to the organelle via the endoplasmic reticulum courtesy of a bipartite amino-terminal-recognition sequence. The extensive metabolic activities of the apicoplast impose a high demand for antioxidant protection. Like most aerobic eukaryotic cells, Toxoplasma possesses mitochondrially targeted SODs and peroxidases to safely dispose of oxygen radicals that are generated by cellular respiration and metabolism. Additionally, a subset of those enzymes is shared between the two organelles by an unusual mechanism of bimodal targeting. TgSOD2 and the thioredoxin-dependent peroxidase TgTPX1 exhibit a bipartite N-terminal targeting sequence similar to conventional apicoplast-targeting, however the nature of the signal peptide influences the destination to both the mitochondrion and the apicoplast. This phenomenon of bimodal targeting is not restricted to enzymes involving in the detoxification of radical oxygen species but also extends to other classical metabolic enzymes such as the tricarboxylic cycle member aconitase. In addition to a glyceraldehyde-3-phosphate dehydrogenase, the presence of aconitase and isocitrate dehydrogenase in the plastid organelle are likely to produce the reducing power necessary for the metabolic activities. Furthermore, aconitase also contributes to the 2-methylcitric cycle, a pathway known to use the catabolism of amino acids to generate pyruvate in bacteria and fungi. Interestingly, only the coccidians but not the piroplasmida or haemosporida have acquired the complete set of genes involved in this pathway by lateral transfer (PrpE, C, D and B). The importance of this pathway has carbon source for this obligate intracellular parasite are under investigation.

634

**Disruption of Host Microtubule Cytoskeleton and Centrosome Delocalization by Intracellular *Toxoplasma***

J. D. Romano, N. Bano, I. Coppens; Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

*Toxoplasma gondii*, an obligate intracellular parasite, actively invades mammalian cells forming a parasitophorous vacuole (PV) from which host plasma membrane proteins are excluded. The PV of *Toxoplasma* exhibits a steady-state distribution, appearing stationary in the perinuclear region of host cells. First, we wanted to determine if the centripetal migration of *T. gondii* inside cells involves host microtubules, and second to explore if the PV perinuclear localization alters the global architecture of the host cytoskeleton. Live cell imaging along with time-lapse confocal microscopy reveals that the parasite moves directionally towards the host nucleus within 30-min and is associated with host microtubules. However, host microtubules are not involved in the parasite intracellular locomotion and host motor proteins (e.g. dynein and kinesin) do not associate with the PV. As the parasite approaches the nucleus, host microtubules appear to extend towards the *Toxoplasma* PV, wrapping around the PV and completely surrounding each individual vacuole. The microtubule overcoating of the PV is initiated at the host nuclear surface about 4-h post-infection and becomes more prominent as parasite division proceeds. Early on, the PV is randomly localized with regards to the host microtubule-

organizing center (MTOC). The PV membrane rapidly forms long extensions supported by host microtubules towards the host MTOC. This results in delocalization of the MTOC from the host perinuclear region to the PV and a stable anchorage of the MTOC to the PV membrane 24-h post-infection. In conclusion, the perinuclear localization of the PV followed by the rearrangement of the host microtubule network, most likely through the usurpation of the MTOC, may allow the parasite to manipulate host membrane-bound organelle trafficking, and thereby to scavenge nutrients.

635

### *Toxoplasma gondii* βCOP Localization and Interactions During Infection

S. S. Smith, J. Moran, K. M. Hager; Biological Sciences, University of Notre Dame, Notre Dame, IN

*Toxoplasma gondii* is the obligate intracellular protozoan parasite that causes Toxoplasmosis in immunocompromised individuals. *T. gondii* has three unique invasion organelles and a residual chloroplast organelle, the apicoplast. Since protein secretion plays an essential role in invasion and maintenance of infection, we are characterizing proteins implicated in regulating protein secretion. In particular, we are studying *Toxoplasma gondii* β-COP like protein (*Tg*βCOP). βCOP has been shown to be involved in protein transport in the early secretory pathway and more recently has been implicated in a variety of protein-protein interactions. Immunofluorescence assays (IFA), immunoprecipitation (IPs), and bioinformatic studies support both novel and conserved roles for *Tg*βCOP. Bioinformatic analysis of *Tg*βCOP reveals a novel C-terminal sequence unique to Apicomplexan parasites, indicating an Apicomplexan specific function. We expect that this novel sequence is either responsible for the differential localization of *Tg*βCOP and/or it serves as a site for a novel protein-protein interaction. In support of the latter hypothesis, IP analysis indicates that *Tg*βCOP interacts with a calcium sensitive molecule. IFA data supports this interaction revealing that *Tg*βCOP localization is sensitive to changes in pH and calcium. This is noteworthy given that a calcium surge in the parasite precedes invasion of the host cell. After invasion and establishment of infection, *T. gondii* parasites replicate asexually. Therefore it is significant that 24 hours after infection ~90% of dividing parasites shows that TgβCOP not only associates with the Golgi (as expected) but also encircles the apicoplast. Based on these results, we hypothesize that *Tg*βCOP has another function besides its role in Golgi to ER retrograde transport. Specifically, during the course of the lytic cycle, we expect that *Tg*βCOP is able to function in transport of materials either to or from the apical organelles and the apicoplast during parasite biogenesis.

636

### Implication of TGF- beta on *Trypanosoma cruzi* infection

M. C. Waghabi,[1] L. M. Lima,[1] W. M. Degrave,[1] M. N. C. Soeiro,[2] M. Keramidas,[3] S. Bailly,[3] J. Feige,[3] T. C. AraújoJorge[2]; [1]Biochemistry and Molecular Biology, Fiocruz, Rio de Janeiro, Brazil, [2]Ultra Structure and Cell Biology, Fiocruz, Rio de Janeiro, Brazil, [3]EMI 0105, Department of Molecular and Structural Biology, INSERM, Grenoble, France

Fibrosis is one of the most important characteristics of the Chronic Chagasic Cardiomyopathy and the anti-inflammatory cytokine, TGF- beta, plays a central role in the development of fibrosis by regulating the synthesis of extracellular matrix. In the present work, we studied the possible role of TGF- beta as an inductor/ regulator agent of the chagasic cardiac remodeling, especially fibrosis. Our data regarding the chronic chagasic patients indicated an association between TGF- beta and fibrosis development. Furthermore, we found important alterations in the pattern of Cx43 distribution in chagasic patients heart cells and within cardiomyocytes cell cultures incubated with exogenous TGF- beta, which could be related to the altered impulse conduction and arrhythmias in the heart. As TGF- beta has been concerned in the invasion of host cells by *T. cruzi*, we next studied the parasite ability to activate host TGF- beta. We observed that *T. cruzi* is able to activate latent TGF- beta in a dose-response manner being also temperature-dependent. Moreover, we observed that *T. cruzi* was able to uptake host TGF- beta, to stock it during intracellular proliferation and possibly use it as a signaling mediator to trigger differentiation into trypomastigotes. In a general context, the *T. cruzi* ability to accumulate and activate TGF- beta could be related to different mechanisms directly or indirectly involved in the development of Chagas disease. Our data suggest that the presence of TGF- beta in the heart can collaborate in the fibrosis formation and arrhythmias reported during the chagasic cardiomyopathy besides contributing for the maintenance of the parasite load in the host tissues.

637

### *Giardia lamblia* Adhesive Force is Insensitive to Surface Treatments

W. R. Hansen,[1] S. C. Dawson,[2] W. Z. Cande,[2] D. A. Fletcher[3]; [1]Biophysics, UC Berkeley, Berkeley, CA, [2]MCB, UC Berkeley, Berkeley, CA, [3]Bioengineering, UC Berkeley, Berkeley, CA

*Giardia lamblia*, a protozoan parasite of the small intestine, causes the gastrointestinal disease giardiasis. *Giardia* adhere to microvilli of epithelial cells in the host small intestine, but the exact mechanism of cellular attachment is unknown. It is likely that attachment and virulence are closely linked by the requirement that trophozoites withstand the periodic pressure of peristalsis. Adhesion of *Giardia* is thought to be mediated primarily by the ventral disc, a 7μm-diameter spiral of microtubules and associated proteins located on the anterior portion of the cell, but the magnitude and mechanism of adhesion is unknown. In this study, we used a centrifuge-based force measurement technique to quantify attachment forces *in vitro* to a variety of surface treatments. In comparison to clean glass surfaces, we found no significant differences in the detachment forces of cell populations from positively charged poly-lysine, hydrophobic tridecafluoro tetrahydrooctyl trichlorosilane, or inert polyethylene glycol (PEG) coated glass. Our results indicate that an attachment mechanism based solely on specific (ligand-mediated) binding is not used by cells in culture and that a mechanism relying solely on electrostatic charge is unlikely. Ultimately, a better understanding of attachment mechanism may aid in the development of specific methods for inhibiting adhesion as a treatment of *Giardia* infections.

638

### Analyze of *Giardia lamblia*-Host Cell Interaction

A. Gadelha, R. Travassos, L. Monteiro-Leal; Department of Histology and Embryology, State University of Rio de Janeiro, Rio de Janeiro, Brazil

*Giardia lamblia* is a flagellated protozoan, which is the causative agent of giardiasis. This cell exerts its parasitism adhering to the host intestinal epithelium. Trophozoites present stable microtubular structures in their cytoskeleton, such as flagella and ventral disc, which participate in the locomotion and adhesion of the cell. In order to understand the dynamic process related to the interaction between *G. lamblia* and intestinal cells, techniques such as video-microscopy have been used by our group. The observations of *Giardia* adhesion, body movements and morphology are allowing us to analyze the effects of several chemicals agents, such as metronidazole and furazolidone, in the attachment of trophozoites to host

cell. We have been using the following methodology: parasites are cultivated in TYIS-33 medium and intestinal cell are grown in DMEM medium. After the formation of monolayer by IEC-6, the parasites are added to the culture medium of these cells, in the presence or absence of drugs. After 24h of treatment, furazolidone showed a reduction of 80% in the adherence of *Giardia*, while metronidazole showed a reduction of only 60%, in the same type of analysis. By video-microscopy, we observed that after 6h and 24h of incubation with metronidazole, the trophozoites showed major modifications in the dorsal surface, despite the fact that some parasites were still able to adhere to the intestinal cells. The parasites treated with furazolidone for a period of 6h, showed slight modifications in their surface. However, after 24h of treatment of the co-culture, the majority of the cells loses their adherence to intestinal cells and was observed without motion in the supernatant. The data obtained indicated that furazolidone affects more the adhesion of the protozoon than its growth pattern, indicating that, this drug is more effective in reduce the attachment then metronidazole.

639

**Charcterization of the Receptor-binding Domains of *Trichomonas vaginalis* AP65 Adhesin**

A. F. Garcia, J. F. Alderete; Microbiology, UTHSCSA, San Antonio, TX

*Trichomonas vaginalis* is an amitochondriate protist responsible for the number one, non-viral sexually transmitted disease. The ability of trichomonads to cytoadhere to vaginal epithelial cells (VECs) is preparatory to successful infection of women. *T. vaginalis* specifically binds to host VECs via the surface expression of at least four adhesin proteins (AP65, AP351, AP33 and AP23). In this work, we focus our studies on determining the binding epitope of the prominent AP65 adhesin. Based on previous published data showing the interacting domains with antibodies generated against AP65, we subcloned different overlapping fragments of AP65 in a pET26b (+) vector containing a C-terminal His-Tag for purification. Cloning of the fragments was verified by PCR. Expression in *Escherichia coli* was then achieved by IPTG induction. The expressed recombinant subcloned proteins were confirmed by detection in nitrocellulose blots after SDS-PAGE and blotting using both specific monoclonal and polyclonal anti-AP65 antibodies. Then each recombinant protein was tested for binding activity using an established host cell binding assay. Furthermore, synthetic overlapping 25-mer peptides containing the N-terminal region of AP65 were used to conduct binding inhibition studies. The data suggest that the cell surface interactive domain of AP65 resides on the N-terminal residues.

640

***Trichomonas vaginalis* Genes Induced upon Contact with Human Vaginal Epithelial Cells**

A. S. Kucknoor, V. Mundodi, J. F. Alderete; Microbiology, UT Health Science Center, San Antonio, TX

Host parasitism by *Trichomonas vaginalis* is complex, and the adhesion to vaginal epithelial cells (VECs) by trichomonads is preparatory to colonization of the vagina. The objective of this study was to identify genes that are induced due to signaling of trichomonads following adherence. In order to identify the genes that are up-regulated, we constructed a subtraction cDNA library enriched for differentially-expressed genes from the parasites that were in contact with the host cells. Thirty randomly selected cDNA clones representing the differentially regulated genes upon initial contact of parasites with host cells were sequenced. Several genes encoded for functional proteins with specific functions known to be associated with colonization, such as adherence, change in morphology, and gene transcription and translation. Interestingly, genes unique to trichomonads with unknown functions were also up-regulated. Semi-quantitative, reverse transcription polymerase chain reaction (RT-PCR) confirmed expression of select genes. Increased amount of protein was demonstrated by immunoblotting with monoclonal antibody. Finally, we showed by RT-PCR the transcriptional regulation of some genes by iron. To our knowledge, this is the first report addressing the differential regulation of *T. vaginalis* genes immediately upon contact with VECs.

641

**Antisense RNA Inhibition of *Trichomonas vaginalis* AP33 Adhesin Gene Expression**

V. Mundodi, A. S. Kucknoor, J. F. Alderete; Microbiology, UTHSCSA, San Antonio, TX

*Trichomonas vaginalis* is the causal agent of trichomonosis, the most prevalent non-viral sexually transmitted disease worldwide. The key step in the initiation and establishment of infection is the adherence of the parasite to host epithelial cells. Adherence is known to be mediated by the coordinated expression of surface protein adhesins (AP65, AP51, AP33, and AP23). Earlier, we showed for the first time the antisense RNA-mediated inhibition of AP65 gene expression in *T. vaginalis*. Abolishing AP65 gene expression resulted in poor parasite adherence to host cells. AP33 is known to be another adhesin, and, like AP65, has sequence identity to an enzyme also localized to hydrogenosome organelles involved in energy generation. Therefore, AP33 is another member of a family of trichomonad proteins with functional diversity. In order to validate the adhesin function of AP33 by genetic means, our objective was to establish an efficient transfection system for antisense RNA inhibition and silencing of the *ap33* gene expression. We confirmed the cloning into the vector pBS-*neo* in sense and antisense orientations the gene sequence coding for AP33. This gave the plasmids pBS-*neo*AP33S (sense) and pBS-*neo*AP33AS (antisense). *T. vaginalis* parasites were then successfully transfected with AP33S and AP33AS plasmids, and stable transfectants were selected using G418. PCR amplification of the neomycin gene (*neo*) from the transfectants confirmed the presence of plasmids. Western blot analysis of total proteins from 33AS transfectants showed a significant reduction in the amount of AP33 compared to controls. This successful decreased synthesis of the AP33 adhesin is a critical first step toward confirming at a genetic level the role of this protein in adherence and, therefore, parasite virulence.

642

**Glyceraldehyde-3-phosphate dehydrogenase Synthesized by the Amitochondriate Eukaryote, *Trichomonas vaginalis*, is a Highly Immunogenic Surface Expressed Protein**

T. Chang, J. F. Alderete; Microbiology, UTHSCSA, San Antonio, TX

*T. vaginalis* is a protozoan responsible for the number one, non-viral sexually transmitted disease that is responsible for significant adverse health outcomes for both women and men. Adherence to vaginal epithelial cells is a virulence property that is preparatory to host infection. The surface adhesins are proteins that mediate adherence and are members of multigene families encoding proteins with functional diversity, as the proteins are also enzymes that reside within hydrogenosome organelles. A library of monoclonal antibodies (mAbs) was generated, and mAbs were screened for immunoreactivity towards parasite surface proteins using a whole cell-ELISA. Four mAbs were identified that recognized a cDNA clone

expressing recombinant glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The mAbs detected live organisms by indirect immunofluorescence and reacted with chemically-stabilized trichomonads. Analysis by cell fractionation revealed the protein in the membrane fraction. A sandwich ELISA with immobilized patient serum IgG readily bound recombinant GAPDH protein that was then detected by the mAbs, indicating the immunogenic nature of GAPDH in patients. These data are exciting new findings, and the literature is replete with the functional diversity of GAPDH among microbial pathogens and mammalian cells. This work extends our findings on the surface placement of trichomonad enzymes, and GAPDH may likewise be an important immunogenic virulence factor for *T. vaginalis*.

643

**_Mycoplasma hominis_ Infects Clinical Isolates of _Trichomonas vaginalis_ and Affects Growth Rates and Synthesis of Adhesin and Patient Antibody Responses**

J. F. Alderete, T. Chang; Microbiology, UTHSCSA, San Antonio, TX

The number one, non-viral sexually transmitted infection worldwide is caused by the protist *T. vaginalis*, which is responsible for significant adverse health outcomes for both women and men. Adherence by the parasite to vaginal epithelial cells (VECs) is a virulence property, and one surface protein called AP65 is a prominent adhesin mediating adherence. Our objective was to evaluate the presence of mycoplasma in clinical isolates of *T. vaginalis* and to ascertain whether mycoplasma infection affected properties, such as growth rates and synthesis of AP65. PCR using *M. hominis*-specific primers was optimized on known positive and negative controls, and a 330-bp mycoplasma PCR product was found in 44 of 58 fresh isolates (76 %). Interestingly, supernatants of cultures of mycoplasma-infected trichomonads were cloudy compared to supernatants of cured and/or mycoplasma-free cultures. Pellets of cloudy supernatants were capable of reverting PCR-negative to PCR-positive for mycoplasma detection among organisms, indicating the presence of infectious mycoplasma released into supernatants during growth by infected trichomonads. Sera of 97 of 136 patients (71%) tested positive for the presence of anti-mycoplasma antibody by ELISA and immunoblot, which was in agreement with the PCR analysis. Trichomonads cured of mycoplasma had higher rates of growth compared to mycoplasma-infected, parental isolates. Also, immunoblots showed that cured trichomonads synthesized greater amounts of AP65 adhesin than did infected parasites. Importantly, mycoplasma bound purified AP65, suggesting a mechanism for subverting VEC adherence mechanisms for interacting and possibly invading *T. vaginalis* in vivo. In conclusion, this work supports reports by others on mycoplasma infections of *T. vaginalis* and shows mycoplasma affecting growth rates, synthesis of adhesin and host antibody responses.

644

**Micrornas mediate C parvum-induced Upregulation of Toll-like Receptors In Cholangiocytes**

X. Chen, P. L. Splinter, S. P. O'Hara, N. F. LaRusso; Mayo Medical School, Rochester, MN

Recent studies have demonstrated that microRNA (miRNA)-mediated post-transcriptional gene regulation may be involved in a wide range of essential biological events including host-cell defense responses to microbial infection. Toll-like receptors (TLRs) are an evolutionarily conserved family of cell surface pattern recognition receptors that are key contribute to innate immunity by detecting invading pathogens. Using an *in vitro* model of biliary cryptosporidiosis employing freshly excysted *Cryptosporidium parvum (C. parvum)* sporozoites and a human cholangiocyte cell line, we previously demonstrated that *C. parvum* induces TLR2 and 4 upregulation in cholangiocytes. However, the precise mechanisms resulting in *C. parvum*-induced TLR upregulation remain unclear. Here, we tested the HYPOTHESIS that endogenous miRNA-mediated post-transcriptional regulation may mediates *C. parvum*-induced upregulation of TLRs. Using an *in silico* computer-generated database search approach, we identified several miRNAs that potentially target TLR2 and 4 mRNAs, i.e., miR-9 and miR-337 for TLR2 mRNA and Let-7i for TLR4 mRNA. We next demonstrated expression of miR-9, miR-337 and Let-7i in cultured normal human cholangiocytes by Northern blot and microarray analysis. Moreover, we found that expression of these miRNAs was altered and related to expression of TLR2 and 4 during *C. parvum* infection. Specifically, we designed an anti-sense, 2-methoxy oligonucleotide specific to Let-7i, and showed that reduction of Let-7i significantly increased TLR4 protein expression in cultured cholangiocytes by 2.5 Xfold, confirming that a Let-7i-mediated post-transcriptional suppression pathway of TLR4 exists in cholangiocytes. In addition, we found that Let-7i was decreased in cholangiocytes 3 Xfold after exposure to *C. parvum*. Taken together, our data indicate that: i) the machinery for miRNA-mediated post-transcriptional suppression of TLR expression exists in cholangiocytes; and ii) this regulatory machinery is altered upon *C. parvum* infection and thus, mediates *C. parvum*-induced upregulation of TLRs in cholangiocytes.

645

**The Interaction Between Opportunistic Parasitic Amoebae and Extracellular Matrix Glycoproteins**

B. Rocha-Azevedo,[1,2] G. A. Cabral,[2] F. C. Silva-Filho,[1] F. Marciano-Cabral[2]; [1]Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Dept of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA

Amoebae of the genera *Acanthamoeba* and *Balamuthia* are encountered worldwide. Some species can cause life-threatening infections, mainly in immunocompromised individuals. *Acanthamoeba* spp. are the causative agents of encephalitis, cutaneous lesions, and keratitis. *Balamuthia mandrillaris* is a recently described opportunistic pathogen, which causes encephalitis. The mode that these amoebae cause cytopathogenesis is not known, but it is documented that these protozoa are able to adhere and to destroy mammalian cells. After host cell destruction, it has been postulated that amoebae gain access to a group of biomolecules that are components of the extracellular matrix (ECM) that are important in the progression of protozoan infections. In *Balamuthia* and *Acanthamoeba* infections, they may play a role in the infection process, since these amoebae are invasive microorganisms. The objective of this study was to characterize the interaction of *A. polyphaga*, *A. culbertsoni*, *A. astronyxis* and *B. mandrillaris* with three pivotal ECM glycoproteins (Fibronectin, Laminin-1 and type I Collagen). To quantify these interactions, we performed binding assays using $^{3}$H-radiolabelled amoebae. We observed that amoebae are able to attach to ECM molecules with different avidity (binding on laminin >collagen >fibronectin). We determined that different species of *Acanthamoeba* exert differential specific binding to ECM glycoproteins. *Balamuthia*, which attach poorly in plastic flasks, demonstrate a major increase in attachment when the plastic substratum is coated with Collagen and Laminin. Morphological differences were observed by light and scanning electron microscopy depending on the type of substratum. Numerous lamelipodia were observed for amoebae adhered to laminin, while an increase of acanthapodia was observed for amoebae adhered to collagen and fibronectin. Differential binding to ECM glycoproteins may articulate a mode by which opportunistic amoebae effect

cytopathogenesis. Financial Support: CAPES-MEC (PDEE-BEX: 0158/05-9), CNPq, FUJB-UFRJ, MCT-PRONEX and NIH ( DA 05832, DA 05274).

646

**Roles of Toll-like Receptor 2 and 4 in In Vitro Response of Macrophages to *Pneumocystis carinii***

C. Zhang, S. Wang, M. E. Lasbury, P. J. Durant, C. Lee; Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN

*Pneumocystis carinii* (*P. jiroveci* for that infects humans) is a major opportunistic fungal pathogen in immunocompromised hosts. The innate immunity to this organism is not well understood. In this study, we showed that normal mouse alveolar macrophages (AMs) respond to *P. carinii* organisms by activating the nuclear translocation of NF-kB, leading to production of proinflammatory cytokines TNF-α and MIP-2. Using gain-of-function studies with transiently transfected HEK293 cells, we found that TLR2, but not TLR4, confers HEK293 cells NF-kB response to *P. carinii*. TNF-α and MIP-2 productions in response to *P. carinii* in mouse alveolar macrophages were inhibited by monoclonal antibody T2.5, which specifically blocks the ligand-binding ability of TLR2. AMs from TLR2-/- mice showed little increase in TNF-α and MIP-2 mRNA levels upon *P. carinii* stimulation, similar to control AMs that were stimulated with only sterile saline. Taken together, these results show that TLR2 plays an important role in the innate immune response to *P. carinii.*

647

**Gene Expression Profile of the Nematode *Heterodera glycines* Throughout Development**

A. A. Elling,[1] M. Mitreva,[2] X. Gai,[3] M. Dante,[2] J. P. McDermott,[4] T. R. Maier,[4] A. Kalyanaraman,[5] S. Aluru,[5] R. S. Hussey,[6] E. L. Davis,[7] J. P. McCarter,[2] T. J. Baum[4]; [1]Interdepartmental Genetics Program, Iowa State University, Ames, IA, [2]Genome Sequencing Center, Washington University School of Medicine, St. Louis, MO, [3]L.H. Baker Center for Bioinformatics and Biological Statistics, Iowa State University, Ames, IA, [4]Department of Plant Pathology, Iowa State University, Ames, IA, [5]Department of Electrical and Computer Engineering, Iowa State University, Ames, IA, [6]Department of Plant Pathology, University of Georgia, Athens, GA, [7]Department of Plant Pathology, North Carolina State University, Raleigh, NC

The soybean cyst nematode *Heterodera glycines* is an obligate biotrophic plant-parasitic nematode which causes annual soybean yield losses of $800 million in the USA alone. Genomic approaches to characterize the gene expression profile of parasitic nematodes open new ways to identify genes that are central to successful parasitic relationships and that might be exploited as nematicide targets. To better understand the developmental biology of this nematode species, we generated and analyzed 20,109 5' expressed sequence tags (ESTs) covering all major life stages (eggs, pre-parasitic J2, parasitic J2, J3, J4, adult females). The transcripts were grouped into 7,734 contigs and 6,883 clusters, possibly representing 36% of all *H. glycines* genes assuming approximately 19,000 genes as in *Caenorhabditis elegans*. The GC content of the EST set studied was 49% with more than 10 million total nucleotides. To classify the ESTs by function, we used the Gene Ontology hierarchy as well as the KEGG database. Sequence alignments revealed *H. glycines* genes with similarity to *C. elegans* genes for which known RNAi phenotypes exist. Furthermore, we were able to identify putative nematode-specific and *Heterodera*-specific genes as well as genes that might have been acquired through horizontal gene transfer from prokaryotes. In summary, this dataset greatly increased the available sequence information for this important nematode species and reveals for the first time a gene expression profile throughout development for a plant-parasitic nematode.

648

**Association of Rab GTPases with the *Salmonella*-Containing Vacuole**

A. C. Smith,[1,2] X. Jiang,[2] J. T. Cirulis,[3,4] W. D. Heo,[5] T. Meyer,[5] J. E. Casanova,[6] M. A. Scidmore,[7] J. H. Brumell[1,2]; [1]Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON, Canada, [2]Infection, Immunity, Injury and Repair, Hospital for Sick Children, Toronto, ON, Canada, [3]Department of Biochemistry, University of Toronto, Toronto, ON, Canada, [4]Cardiovascular Research, Hospital for Sick Children, Toronto, ON, Canada, [5]Department of Molecular Pharmacology, Stanford University, Stanford, CA, [6]Department of Cell Biology, University of Virgina School of Medicine, Charlottesville, VA, [7]Department of Microbiology and Immunology, Cornell University, Ithaca, NY

The intracellular pathogen *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) resides in a membrane bound vacuole, the *Salmonella*-containing vacuole (SCV). Previous studies suggest the bacteria modify trafficking of the SCV, acquiring a subset of late endosomal/lysosomal proteins, avoiding fusion with the lysosome, and establishing a niche for replication. Approximately 6-8 hours after invasion, *S.* Typhimurium form filamentous membrane structures from the SCV called *Salmonella*-induced filaments (Sifs). To examine SCV trafficking we studied its interactions with intracellular markers. There are over 60 Rab GTPases, each interacting with effector proteins to coordinate vesicular traffic. We studied the interaction of 44 Rabs with two strains of *S*. Typhimurium: wildtype and a strain (Δ*invA/inv*) that traffics to the lysosome. We looked for co-localization of Rabs with the SCV at various times within three hours of invasion, as well as with Sifs. Many differences were seen between the two strains, one of which being an accumulation of the endocytic recycling marker Rab11 around wildtype bacteria and not Δ*invA/inv*. We determined that the wildtype SCV interacts with multiple regulators of endocytic recycling, and this is required for efficient SCV maturation. Our findings demonstrate the complex and dynamic nature of the SCV and the utility of performing a 'Rab screen' to understand phagosome modulation by intracellular pathogens.

649

**Development of a Polarized Epithelial Cell Model to Study the Role of Akt/PKB in Salmonella Enterica Interactions with the Intestinal Epithelium**

R. Ireland, O. Steele-Mortimer; LICP, NIAID/NIH, Hamilton, MT

The facultative intracellular pathogen *Salmonella enterica* is a common cause of gastroenteritis. Virulence is dependent on the ability of *S. enterica* to invade intestinal epithelial cells, which is mediated by a type III secretion system (TTSS) encoded on *Salmonella* Pathogenicity Island 1 (SPI1). Several effector proteins translocated by the SPI1 TTSS promote massive actin rearrangements leading to plasma membrane ruffling and subsequent engulfment of the bacteria. One of these effectors, the inositol phosphatase SopB/SigD, also mediates sustained activation of the pro-survival kinase Akt/PKB. Studies using HeLa cells have demonstrated that SopB-dependent Akt activation delays apoptosis and protects infected

cells from chemical induction of apoptosis. To investigate the role of Akt activation in intestinal epithelial cells we have developed a model using the C2Bbe1 cell line, a clonal derivative of human Caco-2 cells. C2Bbe1 cells grown on collagen-coated filter inserts in defined (serum free) media produce uniform differentiated monolayers with tight junctions and well-differentiated brush border membranes within a few days. We found that *S. enterica* serovar Typhimurium invasion of these cells is SPI1-dependent, involves rapid transient effacement of microvilli and plasma membrane ruffling and is associated with sustained phosphorylation of Akt. A mutant lacking SopB invades C2Bbe1 cells but without inducing Akt phosphorylation. As in HeLa cells, SopB-dependent Akt phosphorylation is associated with decreased caspase-3 activation and protection against camptothecin-induced apoptosis. To further characterize the role of Akt activation in intestinal epithelial cells we used siRNA to selectively deplete the two isoforms, Akt1 and Akt2, which are expressed in epithelial cells. The results suggest that the SopB-dependent pro-survival signal is mediated primarily by Akt2.

650

**Perturbation of the Endocytic Pathway Affects Expression and Translocation of Salmonella-effector Proteins in Cultured Epithelial Cells**

P. Fuentes, D. Drecktrah, L. A. Knodler, O. Steele-Mortimer; LICP, NIAID/NIH, Hamilton, MT

The ability of *Salmonella enterica* to survive and replicate within host cells is crucial for pathogenesis and is mediated by a number of virulence factors. In particular, two Type III secretion systems (TTSS) translocate effector proteins directly into the host cell. Effectors translocated by the *Salmonella* Pathogenicity Island 1 (SPI1) TTSS mediate invasion of non-phagocytic cells while those translocated by the SPI2 TTSS are required for intracellular survival. Biogenesis of the *Salmonella*-Containing Vacuole (SCV) involves multiple interactions with the endocytic pathway and is mediated by TTSS effectors. However, the nature of these interactions and the intracellular signals that determine TTSS expression and function have not been fully elucidated. Here we have investigated the effect of perturbing the endocytic pathway, by either inhibiting the vacuolar proton pump (vATPase) or depolymerizing microtubules, on the expression of SPI1 and SPI2 genes as well as on effector translocation. Changes in intracellular gene expression were analyzed by quantitative PCR at 0 - 8 h post invasion. In addition, a destabilized green fluorescent protein (GFP)-based assay was used to determine promoter activity in individual intracellular bacteria. To study translocation of SPI1 and SPI2 effectors into the host cell the calmodulin-dependent adenylate cyclase domain (Cya) of the *Bordetella pertussis* cyclolysin was used as a reporter protein. We found that, in infected HeLa cells, the expression profile of *Salmonella* SPI1 and SPI2 genes was significantly affected by perturbation of the endocytic pathway. Furthermore, efficient effector translocation, particularly of SPI2 effectors, was dependent on vATPase activity. These findings are allowing us to better characterize the role of the endocytic pathway in determining intracellular virulence gene expression and SCV biogenesis.

651

**Helicobacter Pylori Induces a Calcium-dependent Modulation of Actin Cytoskeleton by Activation of Calpain I**

Z. Guo,[1] X. Ding,[1,2] T. Ward,[1] B. Liu,[3] Z. Zhu,[3] X. Yao,[1] A. Shaw[1]; [1]Physiology, Morehouse School of Medicine, Atlanta, GA, [2]Beijing Univ. of Medicine, Beijing, China, [3]Shanghai Institute of Digestive Surgery, Shanghai, China

Helicobacter pylori is one of the most wide-spread bacterial pathogens and implicated in the pathogenesis of gastritis, gastric ulcer, and gastric cancer. Helicobacter pylori targets to gastric glandular epithelial cells and initiates a variety of cellular signaling events to abrogate cell physiology However, the underlying pathogenic mechanisms remain elusive. Our previous demonstration of an inter-relationship between calpain I and ezrin in gastric injury propelled us to examine the possible role of calpain I activation in Helicobacter pylori infection. Here we show that treatment of Helibobacter pylori mobilizes intracellular calcium in cultured gastric parietal cells. The rise in intracellular calcium is contributed by the influx of extracellular pool and the release from the internal store. The elevation of intracellular calcium resulted in an activation of calpain I judged by autolysis of the large subunit of the enzyme and proteolysis of ezrin. This proteolyses were minimized by either pre-treatment of calcium chelator BAPTA or calpain I inhibitor E64, validating the involvement of calcium-dependent proteolysis. Interestingly, Helicobacter pylori-induced calpain I activation induced the reorganization of apex and alterations in cell morphology due to the loss of ezrin. This ezrin-deficient morphological alteration was validated by an ezrin siRNA experiment in which depletion of ezrin resulted in defects in the formation of canalicular apical membranes in gastric parietal cells. Significantly, ectopic expression of ezrin rescued the morphological alteration induced by Helicobacter pylori treatment. We reason that Helicobacter pylori disrupts gastric epithelial cell morphology and physiology by activation of calpain I.

652

**Co-option of Eukaryotic Lipid Bodies by *Chlamydia trachomatis***

Y. K. Budigi, R. H. Valdivia; Molecular Genetics & Microbiology, Duke University Medical Center, Durham, NC

Lipid bodies (LBs) are ubiquitous lipid-rich organelles found in all eukaryotes. While traditionally LBs were regarded as passive neutral lipid storage organelles, recent studies have revealed that LBs are highly dynamic organelles that are intimately involved in lipid, membrane trafficking and cell signaling. During a screen for proteins from the obligate intracellular bacterial pathogen *Chlamydia trachomatis* that are tropic for eukaryotic organelles, we identified four proteins that are recruited to eukaryotic LBs. *C. trachomatis* resides within a membrane-bound compartment that evades fusion with lytic compartments, yet readily acquires host-derived phospholipids, cholesterol and sphingolipids by mechanisms that are poorly understood. We have found that LBs are recruited to the surface of the chlamydial vacuole and that infection results in proliferation of LBs. Analysis of the LB proteome by 2D-DIGE revealed that >40 proteins were altered during infection. Furthermore, pharmacological inhibition of LB biogenesis results in impaired chlamydial growth. Our findings provide evidence for a novel mechanism of organelle subversion wherein *Chlamydia* recruits LBs and co-opts their function for survival. We propose that *Chlamydia* may use LBs as a non vesicle-mediated pathway for lipid acquisition. Alternatively it is possible that this represents an example of “organelle mimicry” where the chlamydial vacuole is protected from the endolysosomal system by being perceived as an ‘LB’ by the host. Molecular characterization of LBs during *Chlamydia* infection presents a unique opportunity to explore the functions of these understudied organelles.

653

**A Comparison of Two High-Throughput Assays for FtsZ Inhibitors**

R. Baliga, D. Guo, N. Tomasevic, Z. Jia, E. DeHostos, K. Brejc, R. Sakowicz, S. Lichtsteiner, D. W. Pierce, J. T. Finer; Assay Development, Cytokinetics, South San Francisco, CA

The FtsZ protein from E.Coli plays a critical role in cell division and therefore represents an attractive target for antibacterial drug development. Previously, a filtration based assay using fluorescently labeled FtsZ mutant T65C has been used to identify inhibitors of FtsZ polymerization by Wang et al (1). Here we describe a comparison between two high-throughput homogeneous assays with simpler liquid handling requirements. The assays are based on binding of GTP by FtsZ and its subsequent hydrolysis, which are essential steps in the FtsZ polymerization/depolymerization cycle. The GTP binding assay uses fluorescence polarization to quantitate the extent of binding of a fluorescent GTP analog to FtsZ. The GTPase assay employs an absorbance-based coupled enzyme system to quantitate the amount of GDP released as a function of time. Both assay methods were developed as high-throughput screens and applied to a library of approximately 20,000 small molecules with generally drug-like properties. Using the GTPase, we were able to identify several FtsZ inhibitors of modest potency (<50 uM). The GTP binding assay is more successful for the slow polymerizing FtsZ's from Mycobacterium tuberculosis and Bacillus subtilis. Using light-scattering assays, we found that inhibitors identified from the two assays do have demonstrable effects on the polymerization/depolymerization of FtsZ and may provide useful starting points for lead optimization against this target. (1) Wang J, Galgoci A, Kodali S, Herath KB, Jayasuriya H, Dorso K, Vicente F, Gonzalez A, Cully D, Bramhill D, Singh S., J. Biol. Chem. 278(45) 44424-8.

654

### Modeling Virus Competition Dynamics Within a Single Host

C. L. Ball, D. Coombs; Mathematics, University of British Columbia, Vancouver, BC, Canada

Viruses that cause chronic infections, such as HIV and hepatitis C virus, are genetically highly variable due to a high rate of error during replication. This high rate of mutation allows for the evolution of multiple strains within a host and leads to competition between strains, with only the fittest surviving. Viral fitness can be measured as the expected number of virions produced over the lifetime of an infected cell. A rapidly replicating virus will produce large numbers of virions quickly, but host cell death is increased due to the damaging effects of viral replication. It is natural to assume that the rate of cell death will increase as a function of increasing viral replication. Based on these assumptions, we can find the optimal rate of viral replication.[1] We have developed a mathematical model that allows the virus to mutate within the host, letting the strains vary only in their replication rate. We have found that the system will eventually evolve to the optimal strain, but virus strains that replicate more rapidly than optimally will always dominate during the initial stages of infection. Additionally, during this initial period the viral load reaches an extremely high level. This is important because it has been shown that transmission rates between hosts are highest when the viral load is high. We are extending our model to allow transmission between hosts, while still allowing the virus to evolve within each host. The purpose of our research is to determine which strains are the most effective at transmitting between hosts and to determine the distribution of virus strains within a population. Footnotes [1]Gilchrist, M.A., Coombs, D. and Perelson, A.S. (2004). Optimizing within-host viral fitness: infected cell lifespan and virion production rate. *Journal of Theoretical Biology.* 229, 281-288.

## Cancer I (655-674)

655

### Temporal Changes in Macrophage Activation May Mediate Lung Tumor Progression

E. F. Redente, A. M. Malkinson; Pharmaceutical Sciences, University of Colorado Heath Sciences Center, Denver, CO

The accumulation of tumor associated macrophages (TAM) enhances the growth of pulmonary adenocarcinomas. Different TAM phenotypes contribute to establishing a tolerant microenvironment that nurtures tumor growth. Classically activated macrophages are triggered by IFN-γ or lipopolysaccharide to become effector cells whose phenotype is characterized by increased production of NO via expression of inducible nitric oxide synthase (iNOS) and initiation of a Th1 lymphocytic response. IL-4 and IL-13 activate macrophages to an alternative state by upregulating arginase I which decreases NO and initiates a Th2 response which promotes tumor growth. Macrophages are a major source of $PGE_2$ during inflammation, and tumor cells undergo paracrine $PGE_2$ signaling to suppress immune responsiveness and allow tumor development. In contrast, prostacyclin ($PGI_2$) antagonizes lung tumor progression in mice. Immunohistochemical analysis of TAM in urethane-induced pulmonary adenocarcinomas in A/J mice exhibits a temporal activation switch in the macrophage population. Macrophages surrounding uninvolved tissue immunostain for arginase in benign adenomas but not in malignant adenocarcinomas. Macrophages have limited iNOS staining in adenomas but increased their iNOS staining in adenocarcinomas. By western blot analysis, iNOS expression was significantly elevated at both time points in tissue adjacent to the tumors compared to age matched control lungs. Arginase I expression was significantly higher in uninvolved tissue near benign tumors than in control animals, but arginase I expression was reduced near malignant tumors, analogous to what was observed by immunohistochemistry. The pulmonary macrophage MH-S cell line can be manipulated by cytokine exposure *in vitro* to exhibit classical vs. alternative activation and provide a model ideal for studying $PGE_2/PGI_2$ production in lung epithelial cells co-cultured with macrophages. Understanding how tumors manipulate, and in turn are regulated by, the macrophage phenotype is important to experimentally modulating tumor progression. (Supported by CA33497 and CA96133)

656

### Inhibition of the Siah-1 Ubiquitin Ligase by a Viral Oncogene Up-Regulates β-catenin in B-lymphoma Cells

J. Shackelford,[1] K. Jang,[2] J. S. Pagano[1]; [1]Lineberger Cancer Center, UNC at Chapel Hill, Chapel Hill, NC, [2]Division of Biological Sciences, Pusan National University, Busan, Republic of Korea

As a multifunctional molecule β-catenin plays important role in cellular processes. The protein levels of β-catenin are tightly regulated by the ubiquitin/proteasome system. We provide evidence that two ubiquitin/proteasomal degradation pathways for β-catenin are active in the same B-lymphoma cells: along with the classical GSK3β-dependent destruction machinery, degradation of β-catenin through Siah-1 ubiquitin ligase is functional in these cells. We show that inhibition of Siah-1 by a ligase-inactive mutant, as well as reduction of Siah-1 expression by siRNA, stabilizes and activates β-catenin in B-cells. Moreover, inhibition of Siah-1-dependent ubiquitination abolishes up-regulation of β-catenin by the principal Epstein-Barr virus oncogene - LMP1. Thus we demonstrate the significance of the endogenous Siah-1-dependent ubiquitin/proteasomal pathway for β-catenin degradation in malignant human cells and its regulation by a viral oncogene.

657

**Vascular Endothelial Growth Factor (VEGF) is Influenced by Hormones of the Sympathetic Nervous System in a Metastatic Prostate Cancer Cell Line**

R. C. Youngblood,[1] N. M. Filipov,[2] P. L. Ryan[1]; [1]Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS, [2]Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS

Psycho-physiological pathways induced by stress, namely activation of the sympathetic nervous system and subsequent release of epinephrine (E) and norepinephrine (NE) have been recognized to influence pathogenic processes, including cancer. VEGF gene expression is known to be affected by the stress hormones, E and NE in ovarian cancer cell lines. Increased levels of VEGF can be indicative of poorer prognoses in patients with cancer. Thus, the goal of this study was to examine the effects of E and NE on VEGF gene expression in a metastatic prostate cancer cell line. To this end PC-3M cells, stably transfected, with a VEGF-*Luc* reporter gene were seeded ($1.0 \times 10^5$ cells/well) in 6-well plates, and cultured for 24 h in media containing charcoal-stripped fetal calf serum, followed by a further 24 h incubation in serum-free media. Cells were then treated with E or NE in replicates of 6 wells at 0, 0.01, 0.1, 1, and 10 μM under serum-free conditions at 70-80% confluency. Cells were harvested at 6, 12, and 24 h post treatment and relative light units (RLUs) were measured using a luminometer. Experiments were repeated 4 times. E, at 1 and 10 μM concentrations, increased ($p<0.05$) VEGF promoter-driven luciferase activity (1537.88 ± 166.53, 1589.5 ± 166.53 RLUs) over that of controls (1156.59 ± 166.53 RLUs) at 12 h and 1 μM NE increased ($p<0.05$) VEGF promoter-driven luciferase activity (1642.33 ± 133.97 RLUs) over that of controls (1286.25 ± 133.97 RLUs) at 12 h. These findings provide experimental evidence that psycho-physiological stress mediators such as E and NE can directly contribute to an increase in VEGF gene expression in prostate cancer cells. [Supported by USDA-ARS G402-21310-001-015, NSF-EPSCoR-0132681]

658

**Fusion with VEGF-C Propeptides Enhances the Biological Activity of VEGF**

S. E. Keskitalo,[1] T. Tammela,[1] T. Karpanen,[1] M. Jeltsch,[1] S. Yla-Herttuala,[2] K. Alitalo[1]; [1]Molecular/Cancer Biology Laboratory, University of Helsinki, Helsinki, Finland, [2]Molecular Medicine Group, University of Kuopio, A.I.Virtanen institute, Kuopio, Finland

The five mammalian vascular endothelial growth factor (VEGF) family members identified to date VEGF, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) are key effectors of physiological and pathological regulation of vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis, and vascular permeability. VEGF is a potent growth factor for blood vessel formation and plays an essential role in this process. Full length VEGF-C has been shown to activate VEGF receptor-3 (VEGFR-3) and to induce lymphangiogenesis, while proteolytic removal of the amino- and carboxyterminal propeptides of VEGF-C yields a mature growth factor that is also able to activate VEGFR-2 and stimulate angiogenesis. The biological properties of the propeptides of VEGF-C are poorly known. We have engineered a chimeric growth factor protein, VEGF-CAC, composed of the amino- and carboxyl-terminal propeptides of human VEGF-C fused to the receptor-activating core domain of human VEGF. We found that VEGF-CAC could be precipitated with soluble forms of VEGFR-1, VEGFR-2, neuropilin-1 and neuropilin-2. Correspondingly, VEGF-CAC supported the growth of Ba/F3 cells transfected with chimeric VEGFR-1/Epo or VEGFR-2/Epo receptors, suggesting that it is a functional ligand. For analysis of the effects of VEGF-CAC *in vivo*, we chose to express VEGF-CAC via adenoviruses in the ear skin of immunodeficient mice. Surprisingly, VEGF-CAC was a more powerful inducer of angiogenesis than even VEGF-$A_{165}$. VEGF-CAC gene transduction resulted in blood vascular leakage, endothelial proliferation, circumferential enlargement of arteries and veins, as well as formation of new blood vessel networks that were largely perfused. In addition, VEGF-CAC induced enlargement of the cutaneous lymphatic vessels, as reported previously with VEGF-$A_{165}$. These results may prove useful for the development of pro-angiogenic therapies in patients suffering from diseases associated with tissue ischemia.

659

**Expression, Regulation and Function of Low Density Lipoprotein Receptor-Related Protein and Urokinase Plasminogen Activator in GBM Cell Lines and the Genetic Modified Human Astrocytes**

Y. Zhao,[1] A. Xiao,[1] R. Abdel-Fattah,[1] S. A. Taylor,[1] R. O. Pieper,[2] I. M. Hussaini[1]; [1]Pathology, University of Virginia, Charlottesville, VA, [2]Neurological Surgery, University of California-San Francisco, San Francisco, CA

Low density lipoprotein receptor-related protein (LRP) is a multifunctional endocytotic receptor that is involved in regulating proteinase activity, which is necessary for cellular invasive processes. One of the well-characterized extracellular proteases is urokinase plasminogen activator (uPA), which plays an important role in the extracellular proteolytic degradation in cancer progression. In this study, we investigated (1) the expression of LRP in pilocytic astrocytoma and glioblastoma multiforme samples (GBM) from patients; (2) the expression of LRP and uPA at mRNA level and protein level in GBM cell lines and in the genetically modified human astrocytes, which resemble human grade II and III astrocytomas and grade IV GBM; (3) the regulation of LRP/uPA expression in NHA clones; (4) the correlation between the expression of LRP/uPA and cell invasion of these cells. Our results reveal that LRP was highly expressed in the pilocytic astrocytomas, but dramatically decreased in GBM; coincidentally, both mRNA and protein of LRP were highly expressed in normal human astrocyte (NHA), E6/E7 and Ras clones, while decreased greatly in EGFR clone and three GBM cell lines, U251, U1242 and U373. The level of uPA in NHA and E6/E7 clone was minimal, but increased in Ras and EGFR clones and in all of the four GBM cell lines. These results were consistent with the immunocytochemical localization of LRP/uPA in these cell lines/clones. Furthermore, the cell invasiveness of Ras and EGFR clones and all four GBM cell lines was significantly increased when compared with NHA. The invasion results correlate well with LRP/uPA expression. Our studies suggest strongly that LRP/uPA may be involved in the tumor cell local invasion, an important characteristic of brain tumor progression.

660

**Nerve Growth Factor Promotes Malignant Breast Epithelial Cell Migration Through a TrkA-dependent Mechanism**

S. Gehler, R. Schmocker, P. J. Keely; Pharmacology, University of Wisconsin, Madison, WI

Breast tumor metastasis is a multi-step process that requires cell migration through interstitial tissue. Neurotrophins are known to regulate survival, differentiation, and outgrowth of neuronal cells, but their effects on non-neuronal cells are not fully appreciated. It has been recently shown that NGF, not normally produced by normal breast epithelial cells, is synthesized and released by malignant breast epithelial cells in vivo (Dolle et al.,

2003). Although neurotrophins have been implicated in regulating the migration of Schwann cells, it is unclear whether neurotrophins have any effect on breast epithelial cell migration. We propose that NGF enhances the migration of transformed breast epithelial (T47D) cells through TrkA activation via an autocrine mechanism. First, NGF enhances collagen-mediated migration in a dose-dependent manner. For instance, 100ng/ml NGF induces a 2.5-fold increase in cell migration. This effect is specific for NGF, as brain-derived neurotrophic factor (BDNF) and NT-3 had no effect on migration. Second, we have demonstrated that NGF and the NGF receptors, TrkA and p75, are expressed on T47D cells using immunofluorescence and Western blot analysis. Third, treatment with a TrkA neutralizing antibody blocked the NGF-mediated increase in cell migration. In addition, the TrkA antibody inhibits migration of non-stimulated cells, which suggests autocrine effects of NGF on migration. These data suggest the novel idea that NGF enhances migration of T47D cells through a TrkA-mediated mechanism. Furthermore, NGF may mediate its effects through autocrine stimulation of T47D cells.

661

**IL-8 is a Potential Biomarker for Chemotherapy of Cervical Cancer**

L. F. Gulyaeva,[1] E. I. Schwartz,[2] V. O. Pustylnyak,[3] M. Bruinmsa,[4] S. E. Krasilnikov[5]; [1]Molecular Carcinogenesis, Institute of Molecular Biology and Biophysics, Novosibirsk, Russian Federation, [2]Department of Pathology, Georgetown University Medical School, Washington, DC, [3]Institute of Molecular Biology and Biophysics, Novosibirsk, Russian Federation, [4]NICHD, NIH, Bethesda, MD, [5]Gynecological Department, Regional Clinical Oncological Hospital, Novosibirsk, Russian Federation

Cervical carcinoma is the second leading cause of cancer deaths in women. In order to increase the efficiency of chemotherapy treatment, it is important to find a potential biomarker, which can be used as a prognosis indicator. Two of the possible candidates are the well-known angiogenic factor interleukin-8 (IL-8) and tumor suppressor gene B-cell leukemia/lymphoma 2 (Bcl-2), a suppressor of apoptosis. The level of gene expression of IL-8 and Bcl-2 in tumor tissues obtained after surgical operation of females with cervical carcinoma was studied. In order to study the possibility to improve survival and decrease toxicity, patients were subdivided into two groups: 19 patients (Group I) received a course of cisplatin, a form of chemotherapy, before surgery and 15 patients (Group-II) were operated on without chemotherapy and were treated with cisplatin after surgery. IL-8 and Bcl-2 expressions were determined by multiplex RT-PCR. Also, PCR-DNA techniques were used to detect the high-risk types of the human papillomavirus (HPV) 16/18. Nearly all of cervical cancer malignancies are the consequence of infection by HPV. Surprisingly, there were no significant differences in Bcl-2 mRNA levels in malignant and adjacent nonmalignant tissues between studied groups. However, IL-8 mRNA level was significantly higher ($p=0.021$) in cervical carcinoma than in adjacent non-transformed cervix tissues of both group patients. Moreover, in tumor tissues of cisplatin-treated patients the level of IL-8 gene expression decreased two fold compared to the untreated tumor. The present data suggests that IL-8 can be used as a potential biomarker in chemotherapy of cervical cancer. In order to further investigate this possibility we will check the correlation between IL-8 and Bcl-2 expressions and the presence of HPV16/18 in tumor tissues of cisplatin-treated and non-treated patients

662

**The Influence of TGF-α on ERE-(Luciferase)-Mediated Gene Expression in the MELN Breast Cancer Cell Line in the Presence of Estrogenic Stimulation**

S. D. Bowers, M. McGee, S. T. Willard; Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS

Estrogen sensitive breast cancer cell lines, including stable photonic or fluorescent cellular models, have been used previously to evaluate the estrogenic activity of various compounds. However, few studies have addressed the interactions of various estrogenic agents (e.g., estradiol (E2), HPTE, etc.) in combination with endogenous factors (e.g., growth factors) that may augment treatment responses in these model cell lines. Transforming growth factor-α (TGF-α) has been shown to have synergistic and inhibitory effects on estrogen response element (ERE)-mediated gene expression in the presence of estradiol and various xenoestrogens depending on the duration of treatment. The objectives of this study were to determine whether the duration of TGF-α treatment would influence the effects of estrogenic stimulation on gene transcription in the ERE-luciferase MELN breast cancer cell line. Cultured, stably transfected MELN breast cancer cells were treated for up to 48 hr with varying concentrations of E2 (1 nM to 1 pM) in the presence or absence of TGF-α (10 ng/ml) or media alone (control). At 1 nM E2, the addition of TGF-α elicited a long term (48 hr) synergistic effect on ERE-mediated gene expression ($P<0.05$), however no short term synergistic effects were noted. Moreover at 10 pM E2, the addition of TGF-α elicited a long term (48 hr) combinatorial effect on ERE-mediated gene expression ($P<0.05$). At 100 pM E2, the addition of TGF-α elicited a short term (6 hr) combinatorial effect on ERE-mediated gene expression ($P<0.05$). These data demonstrate the influence of duration of TGF-α treatment on eliciting synergistic and combinatorial effects on estrogen stimulated ERE-mediated gene expression in the MELN breast cancer cell line. [COBRE-NIH: 5-P2ORR017661-03]

663

**Inhibition of ROS Generation and Cell Migration in Rat Metastatic Prostate Cancer Cells by A3 Adenosine Receptor Agonist**

S. Jajoo, V. Ramkumar; Pharmacology, Southern Illinois University,School of Medicine, Springfield, IL

The adenosine $A_3$ receptor ($A_3AR$) is a heterotrimeric G protein-coupled receptor expressed in variety of tumor cells including prostate cancer cells. Initial evidence suggests that activation of $A_3AR$ suppresses the growth of prostate cancer and melanoma cells in a mouse model. However, the role of $A_3AR$ in cancer cell metastasis is less well characterized. In the present study we test whether $A_3AR$ activation attenuates prostate cancer cell migration and what are the possible mechanism(s) involved in this process. The metastatic rat prostate adenocarcinoma (AT6.1) cells, used in this study, express high spontaneous reactive oxygen species (ROS) generation. Treatment of AT6.1 cells with an $A_3AR$ agonist,IB-MECA, suppressed ROS generation, as determined by the fluorescent probe, 2', 7'-dichlorodihydrofluorescein and confocal microscopy. ROS production was also reduced by compounds AEBSF and DPI, implicating NADPH oxidase as the source of ROS. $A_3AR$ activation also reduced the expression of NADPH oxidase subunits, Rac-1 and gp-91. In addition, IB-MECA reduced the high basal expression of NF-κB in these cells, an important facilitator of ROS-mediated gene expression in cancer cells. ROS plays an important role in the proliferation and metastasis of cancer cells. Hence, we looked for the AT6.1 cell migration using *in vitro* invasion chamber assay in the presence of IB-MECA. 24h treatment of cells with IB-MECA significantly inhibited the cell migration. We conclude that the $A_3AR$ tonically suppresses AT6.1 cells migration *in vitro* and could inhibit prostate cancer metastasis *in vivo*. One likely mechanism underlying inhibition of cell migration is a direct reduction of ROS generation via the NADPH

oxidase pathway and inhibition of gene expression via NF-κB. We propose that the $A_3$AR could serve as novel drug target for suppressing prostate cancer growth and metastasis.

664

**SIKVAV a Laminin-1-Derived Peptide, Binds Alpha3 Integrin and Regulates Protease Activity of a Human Salivary Gland Adenoid Cystic Carcinoma Cell Line through ERK Pathway**

V. Freitas,[1] V. Loureiro,[2] M. A. Juliano,[3] D. Pimenta,[4] A. C. Camargo,[4] M. Hoffman,[5] R. Jaeger[1]; [1]Cell and Developmental Biology, ICB-USP, São Paulo, Brazil, [2]UNIMES, Santos, Brazil, [3]UNIFESP, São Paulo, Brazil, [4]CAT, Butantan Institute, São Paulo, Brazil, [5]Matrix and Morphogenesis Unit, CDBRB, NICDR-NIH, Bethesda, MD

Adenoid cystic carcinoma is a frequently occurring malignant salivary gland neoplasm, with high level of recurrence and metastasis. We have demonstrated that laminin and its derived peptide SIKVAV regulates morphology and protease activity of a cell line (CAC2) derived from human adenoid cystic carcinoma. Here we studied the regulatory mechanisms underlying protease activity induced by SIKVAV in CAC2 cells. MMPs 2 and 9 were immunolocalized in adenoid cystic carcinoma cells *in vivo* and *in vitro*. CAC2 cells were cultured on SIKVAV, to analyze the role of this peptide in regulating protease activity. Zymography of the conditioned medium showed that SIKVAV enhanced secretion of MMPs 2 and 9. We analyzed a putative receptor for SIKVAV in CAC2 cells. Function-blocking antibodies to integrins alpha3beta1 and alpha6beta1 decreased both the adhesion of CAC2 cells to SIKVAV and protease secretion. A similar result was observed when cells were treated with EDTA. RNA interference (RNAi) was also used to silence alpha3 integrin expression. CAC2 cells with decreased alpha3 integrin expression were cultured on SIKVAV, and showed both decreased adhesion and protease activity. Furthermore, we isolated cell surface ligands using SIKVAV affinity chromatography, which were identified as alpha3 integrin by immunoblot. This result was confirmed by solid phase binding assays. We also defined a cell signaling pathway for the effect of integrins in CAC2 cells. A MAPK kinase inhibitor (UO126) decreased the effect of SIKVAV in the secretion of MMP-2 in CAC-2 cells, suggesting that SIKVAV signal is transduced by integrins and the ERK pathway. We propose that SIKVAV regulates protease activity of a human salivary gland adenoid cystic carcinoma cell line through integrin dependant signaling via the ERK pathway

665

**Fra-1-dependent Endocytosis in Mesothelioma Cells Modulates Migration**

M. E. Ramos-Nino, S. Blumen, T. Sabo-Attwood, J. Gell, N. Heintz, B. T. Mossman; Pathology, University of Vermont, Burlington, VT

Gene and protein profiling of mesothelioma cell lines reveal that Fra-1-dependent upregulated expression of endocytosis related genes (caveolin-1, clathrin, and rab27a) are causally related to the rate of migration in these cells. Fra-1, a member of the AP-1 family of heterodimeric transcription factors, is known to be overexpressed in many mesothelioma cells and tumors and has been reported by us to be important in mesothelial cell tumorigenesis, particularly in cell migration. Using microarray, confocal microscopy and standard protein techniques, our studies reveal that several endocytosis-related genes depend on Fra-1 expression and that this relationship accounts in part for the importance of Fra-1 expression in migration of mesothelioma cells. Supported by KO1 CA104159-01 (MERN)

666

**Role of Prohibitin and Glutathione-S-Transferase Pi in Taxol Resistance In Vitro**

S. K. Chatterjee, C. Waghorne, S. Lagasse, G. Najarro, B. R. Zetter; Vascular Biology Program, Childrens Hospital, Harvard Medical School, Boston, MA

Taxol, one of the most successful anti-cancer drugs, is used for the treatment of several metastatic cancers including breast, ovarian and non-small cell lung. Despite Taxol's efficacy, its applicability has been hampered by the development of Taxol resistance, which is observed both in vitro and in patients. P-glycoprotein has been a major contributor in conferring cellular Taxol resistance. Despite the success of P-glycoprotein inhibitors in reversing Taxol resistance *in vitro*, the same inhibitors have failed to reduce Taxol resistance in the clinic. In this study we used a proteomics approach find novel proteins associated with Taxol resistance. Two cell lines and their Taxol resistant variants were used in our study; a lung carcinoma cell line (A549) and its Taxol-resistant subline (A549 TR) and a uterine sarcoma cell line (MES-SA) and its Taxol resistant subline (MES-SA DX5). We found a set of proteins overexpressed in both the drug-resistant sublines compared to their sensitive parental cells. Prohibitin, was found to be a novel protein associated with Taxol resistance. In agreement with this we found that overexpressing prohibitin in the drug sensitive parental cell lines confers Taxol resistance to these cells. In addition, silencing prohibitin expression using siRNA in the Taxol-resistant sublines sensitizes these cells towards Taxol. Differential subcellular localization of prohibitin in the drug-resistant cells compared to the parents correlates with Taxol resistance though importance of prohibitin localization in mediating the resistant phenotype is unknown. Finally, we have shown that silencing both GST-π and prohibitin in concert reverses Taxol resistance significantly. This novel combination therapeutic strategy has implications for the treatment of drug-resistant tumors in the future.

667

**CA125 Tumor Antigen Regulates the Metastatic Potential of the OVCAR-3 Human Ovarian Cancer Cell Line**

C. Rancourt,[1] M. Pinard,[1] M. Boivin,[1] M. Migneault,[1] C. Batiste,[2] C. Theriault,[1] B. Paquette[2]; [1]Microbiologie, Universite de Sherbrooke, Sherbrooke, PQ, Canada, [2]Medecine nucleaire et radiobiologie, Universite de Sherbrooke, Sherbrooke, PQ, Canada

CA125 marker is overexpressed in most epithelial ovarian cancers and used to monitor disease progression. It is a highly glycosylated transmembrane mucin encoded by the *MUC16* gene. Mucins can influence cells metastatic potential. Identification of CA125 function in cells has been hampered by its challenging biochemical nature and large size cDNA. We generated an inhibitor that downregulates CA125 cell surface expression. It consists of an endoplasmic reticulum retained anti-CA125 single-chain antibody (scFv) that acts by sequestering CA125. Stable clones expressing the inhibitor or a control scFv were derived from OVCAR-3 human ovarian cancer cells. FACS analysis validated CA125 cell surface knockdown. To determine whether CA125 influences the OVCAR-3 cells metastatic potential, CA125 knockdown and control clones were compared for their homotypic cell-cell interaction, motility, migration and invasion capabilities. Cell aggregation assays showed that CA125 knockdown impairs cell-cell interaction. Scratch assays demonstrated that CA125 knockdown clones fill more rapidly wounded monolayers.

Migration assays in Boyden chambers confirmed an up to 200-fold increased cell motility. Matrigel-coated Boyden chamber assays showed an augmented invasiness of CA125 knockdown clones. Gene expression profiles of CA125 knockdown clones suggested increased expression of CD44 (10-fold) and proMMP-2 (5 to 10-fold). FACS analysis on non-permeabilized cells confirmed increased CD44 expression. Augmented proMMP-2 expression was validated by zymography which also showed an increased MMP-2 activity. Real-time PCR analysis revealed no change in MT1-MMP expression. Reverse zymography demonstrated a greater TIMP-2 expression supporting the augmented MMP-2 activity observed with zymography. The metastatic process involves the loss of homotypic cell-cell interaction and increases in motility, migration and invasiveness of cells. Our results demonstrate that CA125 cell surface knockdown generates such phenotypes and suggest that CA125 is a negative regulator of the metastatic potential of OVCAR-3 cells.

668

**Auto-Antibody Reactivity to Tumor Markers from both Native Serum Samples and In-Vitro Cell Culture**

M. C. Groll,[1] G. Goodrich,[2] J. D. Hoopes[1]; [1]Quansys Biosciences, Logan, UT, [2]Bioprocessing Inc., Scarborough, ME

Characterization of the relationship of auto-antibodies and antigen is needed to better understand their role in disease. It is hypothesized that auto-antibodies respond differently to antigen from native serum as opposed to cell culture derived antigen. In this study, 2 samples, one derived from sera and the other cell culture, of Alpha Fetoprotein (AFP), Carbohydrate Antigen (CA) 15-3, CA 19-9, CA 125 and Carcinoembryonic Antigen (CEA) donated by Bioprocessing Inc (Scarborough, MA) were screened using 48 random serum samples. 20 nano liter spots of each type of tumor marker, 10 in total, were deposited on the bottom of the well of a 96-well plate. The spots were incubated for 4 hours at 37ºC and then blocked with Quansys Blocker. The serum samples were then diluted in the supplied sample buffer at 1:100 dilution and incubated for 30 minutes at 20ºC on an orbital shaker. After washing each well with the Wash Solution, Quansys Detection was diluted to 1:1000 and added to the plate. This was incubated for 30 mins at 20ºC followed by another wash with the Wash Solution. Quansys Substrate was added and the plate was then imaged using the Fluorchem 8900 (Alpha Innotech Inc.) for 2 minutes. For each of the markers, a residual plot was graphed and analyzed. The $R^2$ of the following systems were AFP (0.64), CA 19-9 (0.94), CA 15-3 (0.18), CA 125 (0.29) and CEA (0.96). The CA 19-9 and CEA showed an acceptable correlation between the natural sera and antigen derived from cell culture. The AFP showed moderate correlation with the poorest correlation being CA 125 and CA 15-3. These poor correlations could potentially be caused by impure antigen samples as well as modified antigen via post-translational modifications.

669

**Multiplexed microELISA Used for Monitoring Renal Cell Carcinoma**

S. Call,[1] A. Ben-Jacob[2]; [1]Quansys Biosciences, Logan, UT, [2]Cache Valley Cancer Treatment Clinic, Logan, UT

Renal cell carcinoma accounts for approximately 2-3% of all adult malignancies. It does not respond well to radiation or chemotherapy; therefore surgery remains the most effective treatment. The objective of this study is to monitor renal cell carcinoma pre and post surgery to detect recurrence or advancement of the disease using multiplexed microELISAs. Through a collaborative project with Dr. Ali Ben-Jacob of the Cache Valley Cancer Treatment Clinic, a sample was taken one-week pre surgery to measure multiple tumor markers and cytokine levels using the Quansys Cancer Array and the Quansys Q-Plex™ Human Cytokine Array. The tumor markers that were measured include AFP, CEA, PSA, IL-6, hCGb, CA125, Prolactin (PRL), and CA19-9. The cytokines that were measured include IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFNγ, TNFα, and TNFβ. A follow-up sample was taken one-month post surgery to observe differences in the protein level expressions. The pre surgery sample yielded a CA19-9 level of 20 U/ml, and IL-6 of 90 pg/ml. The post surgery sample yielded a CA19-9 level of 20 U/ml and IL-6 of <2 pg/ml. Cancerous levels of CA19-9 are expected to be >37 U/ml. The CA19-9 level detected is expected in normal sera. Research has shown that IL-6 could be a potential marker for renal cell carcinoma. Normal levels of IL-6 are expected to range between 5-8 pg/ml. Responses of IL-6 between pre and post surgery samples indicate a definite decrease in IL-6 following the surgical removal of the tumor and kidney. This suggests IL-6 as being a potential marker for renal cell carcinoma. The multiplexed microELISAs prove to be effective tools for monitoring renal cell carcinoma.

670

**Induction of Apoptosis in Tumor Cell Lines by Polyphenolic Compounds Isolated from *Vaccinium macrocarpon***

L. Griffin,[1] S. Rego,[1] E. Correiro,[2] C. Neto,[3] P. Hart[1]; [1]Biology, UMass-Dartmouth, N. Dartmouth, MA, [2]Medical Laboratory Science, UMass-Dartmouth, N. Dartmouth, MA, [3]Chemistry and Biochemistry, UMass-Dartmouth, N. Dartmouth, MA

Polyphenolic compounds, including those present in cranberry fruit, have a cytotoxic effect on a variety of tumor cell lines, and we have investigated the possibility that these cytotoxic effects are mediated through one or more apoptotic pathways. Polyphenolic-rich extracts from cranberry fruit were isolated and fractionated by HPLC. Breast tumor cells, MCF-7, and normal breast epithelial cells, MCF10A, were cultured under standard conditions either in the presence of a total polyphenolic extract (TPE) or fractions of the TPE enriched for distinct classes of polyphenolics. The cellular effect(s) of the compounds were assayed using a fluorescent TUNEL assay and a BrdU incorporation assay. The TPE or ursolic acid induced apoptosis in the MCF-7 cells at a frequency of 92.3% and 98.1%, respectively. These effects appear to be tumor-cell-specific as apoptosis was not induced with either the TPE or ursolic acid in MCF10A cells to a significant extent. Additionally, similar effects were observed in colon tumor cell lines, HT-29 and HCT-116. These results suggest that polyphenolic compounds may hold some chemopreventative or chemotherapeutic promise.

671

**The Mechanism of CRMP-1 in Modulating TNFα-mediated NF-κB Activation**

C. Wang, H. Shih; The Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan Republic of China

Metastasis is the major cause of cancer-induced death. Recent study showed that CRMP-1 (collapsing mediator protein-1) expression suppresses tumor metastasis and is associated with survival rate of patients suffering lung cancer. Furthermore, a defect in the TNFα/NF-κB-mediated IL-8 induction was observed in the CRMP-1 expressing cells. The molecular mechanism of CRMP-1 in downregulating the TNFα/NF-κB signaling and associated cellular events remains unknown. In the present study, we demonstrate that CRMP-1 attenuates TNFα/NF-κB signaling through direct

interaction of NF-κB subunit p65/RelA protein. We first demonstrated that TNFα-induced expression of IL8, cIAP1 and cIAP2 was reduced in CRMP-1 overexpressing cells. Epistatic analysis of TNFα-mediated signaling molecules revealed that CRMP-1 functions as a downstream molecule of p65. Accordingly, TNFα-induced IκBα phosphorylation/ degradation and p65 nuclear translocation were not affected by CRMP-1 expression. *In vitro* and *in vivo* binding studies further indicated that CRMP-1 binds to p65 carboxy terminus and blocks its transactivation and DNA binding activities. Furthermore, depletion of CRMP-1 expression by RNA interference approach increased TNFα-induced NF-κB reporter activation and target gene expression. Lastly, increased or decreased CRMP-1 expression resulted in enhanced or reduced TNFα-mediated cytotoxicity, respectively. In summary, our data provide the mechanistic insight of CRMP-1 in modulating TNFα-mediated NF-κB activation.

672

**L1-CAM, a Novel Target of β-Catenin-TCF Signaling, Transforms Cells and is Expressed at the Invasive Front of Human Colon Cancers**

N. Gavert,[1] M. Conacci-Sorrell,[1] P. Altevogt,[2] T. Brabletz,[3] A. Ben-Ze'ev[1]; [1]Molecular Biology of the Cell, Weizmann Institute of Science, Rehovot, Israel, [2]Tumor Immunology Programme, German Cancer Research Center, Heidelberg, Germany, [3]Department of Pathology, University of Erlangen-Nurnberg, Erlangen, Germany

L1-CAM, a Novel Target of Beta-Catenin-TCF Signaling, Transforms Cells and is Expressed at the Invasive Front of Human Colon Cancers. Beta-Catenin, a protein linking cell adhesion molecules to the cytoskeleton, is also a prominent transcriptional activator during embryogenesis that is aberrantly activated in many human cancers. Stabilizing mutations in beta-catenin, or mutations in its degradation pathway, are among the early and primary changes seen in human colon cancer. Using cell lines and human tissue, we identified the neuronal cell adhesion molecule L1-CAM as a target gene of beta-catenin-TCF signaling in colorectal cancer cells. L1-CAM expression confers increased cell motility, invasion, growth in low serum and transformation. In mice, L1-CAM over-expressing human colon cancer cells were tumorigenic, and when injected into the spleen, exhibited extensive liver metastases. In human colorectal cancer, L1-CAM was exclusively localized at the invasive front of such human tumors, together with ADAM10, in cells also displaying nuclear beta-catenin. L1-CAM expression in human colon cancer cells could therefore be useful for detection, and as a target for colon cancer therapy.

673

**D2 (but not D1 and D3) Dopaminergic Receptor Agonists Inhibit Cholangiocarcinoma Growth by Activation of $Ca^{2+}$/$IP_3$/PKC-gamma**

H. Francis,[1] S. Glaser,[2] S. DeMorrow,[1] J. Venter,[3] R. Reichenbach,[4] R. Summers,[3] B. Green,[3] A. Coots,[1] Y. Ueno,[5] G. Alpini[6]; [1]Scott & White Hospital, Temple, TX, [2]Scott & White Hospital/The Texas A&M University HSC/COM, Temple, TX, [3]The Texas A&M University HSC/COM, Temple, TX, [4]Central Texas Veterans HSC, Temple, TX, [5]Division of Gastroenterology, Tohoku University School of Medicine, Sendai, Japan, [6]Scott & White Hospital/The Texas A&M University HSC/COM/Central Texas Veterans Hospital, Temple, TX

Cholangiocarcinoma is a devastating biliary cancer with limited treatment strategies. Three dopaminergic receptor subtypes (D1, D2 and D3) exist. Activation of D2 dopaminergic receptors by the D2 agonist, quinelorane, inhibits the growth of Mz-ChA-1 cells by activation of $Ca^{2+}$/PKC. **Aims**: (i) To evaluate the effects of D1, D2 and D3 receptors on Mz-ChA-1 growth; and (ii) to further evaluate the mechanisms by which D2 receptors inhibit Mz-ChA-1 growth. **Methods:** We utilized three cholangiocarcinoma cell lines: Mz-ChA-1 cells (from gallbladder); HuH-28 (from intrahepatic ducts) and TFK-1 (from extrahepatic ducts). Receptor expression was evaluated. Time and dose effects were evaluated in cells stimulated with 0.2% BSA (basal) or the D1 agonist, SFK-82958, quinelorane, or the D3 agonist, 7-Hydroxy-DPAT (10, 25, 50, 100 μM for 24 and 48 hours). Mz-ChA-1 cells were stimulated for 48 hours with BSA or quinelorane (25 μM) in the absence/presence of: BAPTA/AM ($Ca^{2+}$ chelator, 5 μM); Gö6976 (PKCα inhibitor, 1 μM); HBDDE (PKCγ inhibitor, 50 μM) or CGP53353 (PKCβI & II inhibitor, 1 μM; obtained from Novartis Pharma AG, Basel, Switzerland). MTS assay was performed. $IP_3$ levels were measured in Mz-ChA-1 cells stimulated with quinelorane. Phosphorylation of PKC α, βI, βII and γ was evaluated by immunoblots from Mz-ChA-1 cells treated with quinelorane for 90 minutes. **Results:** Mz-ChA-1, TFK-1 and HUH 28 cells express all three dopaminergic receptors. Quinelorane decreased growth in Mz-ChA-1 cells at 10, 25, 50, 100 μM for 24 and up to 48 hours. D1 and D3 agonists did not effect growth. Quinelorane inhibition of Mz-ChA-1 growth was blocked only by BAPTA/AM and HBDDE. Quinelorane increased $IP_3$ levels and the phosphorylation of PKCγ. **Conclusion:** Dopaminergic innervation through interaction with D2 agonists may lead to therapeutic strategies for cholangiocarcinoma treatments.

674

**Ephrin-B/EphB Receptor Signaling Controls Morphology and Migration of Murine Melanoma Cells**

N. Yang,[1] E. B. Pasquale,[2] L. B. Owen,[3] I. M. Ethell[1]; [1]Division of Biomedical Sciences, Biochemistry and Molecular Biology Program, University of California, Riverside, CA, [2]The Burnham Institute, San Diego, CA, [3]BioLegend, San Diego, CA

Malignant melanomas are difficult to treat as compared with other types of skin cancer and the incidence of malignant melanomas is increasing faster than that of any other malignancy in the United States. The Eph receptors form the largest family of the receptor tyrosine kinases, which is divided into two classes, A and B, according to their ligand affinity and sequence similarity. Several studies have demonstrated expression of B class Ephs and ephrins in a variety of tumors and suggested a functional relationship between their expression and tumor progression. Here we show an association between expression of EphB receptors, especially EphB4, and the migratory ability of murine melanomas. Highly malignant melanoma cells express the higher levels of EphB4 receptors, which are also phosphorylated on tyrosine suggesting high kinase activity. The melanoma cells expressing highest levels of EphB4 and its ligand, ephrin-B2, migrate faster and show more polarized cell morphology compared to less malignant melanoma cells. Furthermore, we demonstrate that inhibition of ephrin-B/EphB signaling by overexpression of dominant-negative EphB4ΔC, or by treatment with soluble EphB2-Fc, both result in slower migration of highly malignant melanoma cells. Inhibition of EphB4 receptor activity has also pronounced effects on melanoma cell morphology and actin organization. Moreover, the overexpression of active EphB4 in slow migrating melanoma cells significantly enhances cell migration. These data suggest that ephrin-B/EphB receptor signaling contributes to the high migratory ability of melanoma cells most likely by influencing cell substrate adhesion and actin cytoskeleton organization.

## Imaging Technology (675-701)

675

### Specific, Dynamic and Non-Invasive Labeling of Pancreatic Beta-Cells in Reporter Mice Expressing a Proinsulin-EGFP Fusion Peptide

A. Ben-Yehudah,[1] B. Reinhart,[2] C. Navara,[1] J. Kotzuk,[2] S. F. Witchel,[3] G. Schatten,[1] R. Chaillet[2]; [1]Pittsburgh Development Center, University of Pittsburgh, Pittsburgh, PA, [2]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA, [3]Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA

We have developed a non-invasive tool for the detection of directed differentiation of human ES cells into β cells for the treatment of diabetes. Here, we present an efficient, innovative approach for imaging pancreatic β cells. The main physiologic function of β cells is glucose-stimulated insulin secretion. This function is facilitated through the synthesis and storage of insulin in secretory vesicles of β cells, which then release their contents when β cells are exposed to hyperglycemic conditions. To visualize β cells *in vivo* in the mouse, we used targeted mutagenesis techniques to construct a modified *insulin II* (*InsII*) gene allele, $InsII^{EGFP}$ that expresses a proinsulin-EGFP fusion peptide. The EGFP portion of this fusion is entirely within the C-peptide portion of the proinsulin peptide. Prior studies with this construct demonstrated: a) targeting of the fluorescence to vesicles; b) glucose-stimulated secretion of insulin and C-peptide$^{EGFP}$ into the medium; and c) secretatogue-stimulated (KCl and sulfonylurea) secretion of insulin and C-peptide$^{EGFP}$ into the medium. This fusion protein is processed in β cells to insulin and EGFP-tagged C peptide, which are stored together in cytoplasmic secretory vesicles. The large amount of vesicular EGFP-tagged C peptide is evident as a characteristic robust and specific fluorescence pattern in the β cells of $InsII^{EGFP}$ mice. This innovative method of visualizing β cells will be a useful tool in the study of both β cell physiology and the development of the endocrine cells of the pancreas.

676

### A Comparison of Optical Clearing Agents to Enhance Photonic Detection of *Salmonella* through Pig Skin

K. E. Moulton,[1] E. Williams,[1] F. Lovell,[1] P. Ryan,[1] D. Lay,[2] D. Jansen,[3] S. Willard[1]; [1]Department of Animal and Dairy Science, Mississippi State University, Mississippi State, MS, [2]Livestock Behavior Research Unit, USDA-ARS, West Lafayette, IN, [3]Department of Biomedical Engineering, Vanderbilt University, Nashville, TN

The objective of this investigation was to evaluate glycerol (GLY), glucose (GLU), sucrose (SUC), and corn syrup (CS) as agents to optically clear pig skin and increase photonic transference and detection of photon emitting *Salmonella typhimurium* (*S. typh*-lux; transformed with plasmid pAKnlux1), in a laboratory model for *Salmonella* detection in swine. Shoulder pig skin obtained after harvest was processed to remove hair and subcutaneous fat, and the skin measured for thickness. A 96-well plate containing *S. typh*-lux was imaged for 5 min using a photon counting camera (Berthold/Nightowl) as a control reference. Skin (2 mm thick) was placed over the plate containing *S. typh*-lux and imaged 5 min. The skin was then treated for 4 h with varying ratios of GLY, GLU, SUC, and CS in a dose-dependent manner and the plate imaged again 5 min. The percent of photonic emissions (treated or untreated skin relative to no skin controls) from 3 replicates within 8 skin pieces per treatment were used for statistical analysis. Treatment for 4 h with 100% CS increased ($P<0.05$) photonic emissions (21.6 ± 2.0 %) compared to untreated skin, 50% GLY and 50% SUC (0.6 ± 0.2, 17.7 ± 0.8 and 13.6 ± 1.8 % respectively). However, 75% SUC and 50% SUC increased ($P<0.05$) photonic emissions (17.7 ± 1.3 and 17.7 ± 1.3% respectively) compared to 75% GLU, 50% GLY and untreated skin (15.3 ± 1.1, 15.4 ± 0.8 and 1.1 ± 0.1 % respectively). These data indicate GLY, GLU, SUC, and CS may be used as effective optical clearing agents on pig skin when treated for 4 h to allow for an increased detection of emitted photons from *S. typh*-lux through skin. [USDA-NRI grant# 2003-35201-13841; USDA-ARS Biophotonic Initiative# 58-6402-3-0120].

677

### Imaging Subcellular Structure with Hard X-ray

P. Tseng, Y. Hwu; Institute of Physics, Academia Sinica, Taiwan, Taipei, Taiwan Republic of China

We present a experimental evidence that hard X-ray can be used to image the sub-cellular structure at 60 nm resolution. In this study, the live, staining free, plant and animal cells could be imaged by coherent unmonochromatic synchrotron source. X-ray microscopy using un-monochromatic synchrotron source achieve the resolution about 1 um. At this scale, tissue specimens could be imaged at cell level. To localize the distribution of specific protein, we use immunocytochemistry to label the intermediate filaments, vimentin, inside the cytoplasm of HeLa cells. The DAB with nickel enhancement were used as chromogen to increase the absorption contrast. The cell nuclei could be identified because they were free from vimentin staining. When high definition phase zone plate and monochromatic synchrotron source were applied to X-ray microscopy, the images were substantially improved. It permits 60 nm resolution and promises to reach a 25nm resolution. The X-ray emitted from synchrotron source was monochromatized by double crystal monochromator, the photon energy was fixed at 8 keV to optimize the focusing efficiency of the zone plate. The texture of vimentin network at the peri-nuclear area and the vimentin bundles at the cell lamellapodia can be clearly identified. The When compared with visible light and laser confocal microscope, the x-ray microscope provides a superior resolution to both conventional optical microscopes.

678

### Simultaneous Imaging and Analysis of Cellular Dynamics in Three Dimensions in Fluorescence Microscopy

P. Prabhat,[1,2] Y. Chao,[1] S. Ram,[1,3] E. S. Ward,[1] R. J. Ober[1,2]; [1]Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX, [2]Department of Electrical Engineering, University of Texas at Dallas, Richardson, TX, [3]Joint Biomedical Engineering Graduate Program, University of Texas at Arlington / University of Texas Southwestern Medical Center, Arlington / Dallas, TX

Fluorescence microscopy of live cells is an invaluable tool to investigate cellular trafficking pathways. The existing microscope design is very well suited to image fast moving vesicles, tubules and organelles in one focal plane. However, the imaging of such cellular components that move between different focal planes is problematic. Since this would require a focusing device that changes the focal plane of the microscope, which is typically slow. Moreover, in this approach only one focal plane can be imaged at a time. Hence when the cell is imaged at one focal plane, important events could be missed at other focal planes. To overcome these shortcomings, we recently reported a novel modification to the classical microscope design that enables the simultaneous imaging of two distinct focal planes within the sample (1). In our current study, we have extended

this setup to acquire images simultaneously from up to four distinct focal planes. This setup has enabled the study of trafficking behavior that spans a larger depth within the cell than was possible with the earlier configuration. Human endothelial cells were imaged using this setup to study the dynamics of fluorescently labeled FcRn. Trafficking events that would be difficult to study without simultaneous imaging of multiple focal planes will be presented. The visualization and analysis of data acquired from different focal planes poses new challenges. We present software tools that were developed for this purpose. 1. Prashant Prabhat, Sripad Ram, E. Sally Ward and Raimund J. Ober, Simultaneous Imaging of Different Focal Planes in Fluorescence Microscopy for the Study of Cellular Dynamics in Three Dimensions, IEEE Transaction on Nanobioscience, **3**(4), 237-242, (2004) Supported by grants from the NIH (R01 AI39167, R01 AI50747 and R01 AI55556)

679

## Spatial and Temporal Characterization of the Metabolic Response of PC12 Cells to Stimulation: A Two-Photon NADH and Flavoprotein Fluorescence Study

C. H. Keith,[1] E. Porter,[1] A. T. Sornborger[2]; [1]Cellular Biology, University of Georgia, Athens, GA, [2]Math/Engineering, University of Georgia, Athens, GA

It has long been known that the fluorescence of NADH and flavoproteins can be used to estimate the metabolic load being put on cells, Functional imaging measurements of NADH/Flavoprotein fluorescence have focused on imaging relatively large areas of cortex. We are using two-photon fluorescence microscopy of NADH and flavoprotein to measure the metabolic response of the trigeminal ganglion of zebrafish larvae to facial stimulation with irritants (Sornborger et al, Soc. Neurosci. Abstract 2005) In order to rigorously analyze and model these responses of neurons to stimulation, we need to establish the relative contribution of the opening of various channels to their metaobolic response. Therefore, we are stimulating differentiated and non-differentiated PC12 cells with pulses of isotonic high $K^+$ modified PBS, both in the presence and absence of 1 mM $Ca^{++}_o$, as a way of isolating the metabolic loads engendered by ion fluxes consequent to the opening of a variety of different channels. We find that the metabolic response of naïve and differentiated cells to depolarization differs in both magnitude and form, and that the presence or absence of $Ca^{++}_o$ can change their response. We also find that the changes in fluorescence on stimulation is limited to micron-sized punctae within the cytoplasm, consistent with its being largely, if not exclusively, mitochondrial in origin. Therefore, we feel that 2-photon fluorescence microscopy will represent a powerful, non-invasive technique to measure the response of cells to stimuli, both *in vitro* and *in vivo*. Supported by the University of Georgia Research Foundation

680

## $CC^3$: A Stable, Sterile Analog of Poly-D-Lysine that is Optimal for the Culture of Fastidious Cells in Normal and Reduced Serum Media

W. Lu,[1] D. Schroen,[2] H. McIntosh,[2] T. Cummins[1]; [1]Research and Development, Nalge Nunc International, Rochester, NY, [2]Marketing, Nalge Nunc International, Rochester, NY

Poly-D-lysine (PDL) coating of cell culture surfaces improves attachment and growth of certain fastidious cells. However, the PDL surface cannot be considered formally sterile, because current irradiation methods alter the coating function in cell culture. Most PDL surfaces claim a short term shelf life and often require controlled storage conditions. To improve the PDL surface, a non-biological analog of PDL ($CC^3$), a synthetic organic polymer with high amine group density and positive charge in neutral media, has been investigated for its function and stability in cell culture. PC12, HEK293, CHO and BHK cells have been selected, to evaluate the effect of the $CC^3$ surface on cell culture using normal and reduced serum media. Data shows equivalent cell attachment and growth on PDL vs. $CC^3$-coated surfaces when HEK, CHO, BHK and PC12 cells are incubated in normal serum media. HEK, CHO and BHK cells gradually adapt to reduced serum media over several weeks. The attachment, growth and morphology of three cell lines on the $CC^3$ surface in low serum media are similar to those on PDL surfaces. An accelerated aging test shows that the $CC^3$ surface is stable for cell culture for up to 5 years. Importantly, the $CC^3$ surface can be sterilized with gamma-irradiation to SAL $10^{-6}$ levels. Reproducibility of the $CC^3$ surface was demonstrated using 90 plates, including 6 different formats and coated in 3 separate procedures. In all plates, excellent cell attachment and growth was demonstrated. We conclude that $CC^3$ surface is a stable, sterile, non-biological analog of PDL surface, which mimics the function of PDL surface for cell culture in normal and reduced serum media, yet exhibits an extended shelf life, and withstands sterilization treatment.

681

## Tandem Screening of Toxic Compounds on GFP-Labeled Bacteria and Cancer Cells in 96-Well Culture Plates

J. Montoya,[1] A. Varela-Ramirez,[1] M. Shanmugasundram,[2] L. E. Martinez,[2] T. P. Primm,[1] R. J. Aguilera[1]; [1]Biology, University of Texas at El Paso, El Paso, TX, [2]Chemistry, University of Texas at El Paso, El Paso, TX

A 96-well assay has been developed for the rapid screening of potential cytotoxic and bacteriocidal compounds. The assay is based on detection of GFP (Green Fluorescent Protein) in mammalian (HeLa) cells and gram negative (*Echerichia coli*) and gram positive bacteria (*Mycobacterium avium*). Addition of a toxic compound to the GFP marked cells resulted in the loss of the GFP fluorescence which was readily detected by fluorometry. Using this assay, we screened thirty nine distinct naphthoquinone derivatives and found that several of these compounds were toxic to all cell types. In these assays, plumbagin was clearly the most toxic of the test compounds on both the mammalian cells and the two bacterial strains. In addition, two structurally similar compounds, naphthazarin and menadione, were also found to exhibit highly toxic to all cell types. Apart from plumbagin, lapachol was found to be the most toxic to *M. avium*. Two general types of toxic compounds were detected, those that exhibited toxicity to two or all three of the cell types and those that were primarily toxic to the HeLa cells. As the toxicity of these two sets of compounds targets different cell types, it is likely that these compounds will have different modes of action. The tandem analysis of different cell types allowed for the detection of differences in toxicity that would not have been detected if the compounds had been tested on a single cell type. Our results demonstrate that the parallel screening of both eukaryotic and and prokaryotic cells is not only feasible and reproducible but also cost effective.

682

## Low Density Lipoprotein/High Density Lipoprotein on Au(111) Substrate: Atomic Force Microscopy Visualization

A. Segura Salvador,[1,2] N. Batina[1]; [1]Departamento de Quimica, Universidad Autónoma Metropolitana, Mexico City, Mexico, [2]Facultad de Ciencias

Químicas., Universidad Autónoma Benito Juárez de Oaxaca, Oaxaca, Mexico
The low density lipoprotein (LDL) is the vehicle for cholesterol transport inside the human body. It's a positively charged spherical particle with 25 nm of diameter. Inside arteries, can deposit into solid plaque, causing a disease known as atherosclerosis characterized by hardening and loss of elasticity of the walls of the blood arteries. The high density lipoprotein (HDL) is a negatively charged particle, with diameter of 12 nm. It participates in lipid transport, however not in the arterial accumulation. The atomic force microscope (AFM) is an instrument of nanotechnology and surface science, capable to research molecular aggregates with low conductivity, revealing topography of surface at molecular scale. It can operate in variety of media, including aqueous, which has a special importance for visualization of biological surfaces. As majority of samples for AFM analysis, LDL and HDL were fixed at the atomically flat Au(111) substrate. Samples were prepared from diluted physiological solutions: $8x10^{-14}$ M LDL and $8x10^{-13}$ M HDL at 37 °C and visualized by AFM, in air. Individual molecules and very interesting aggregates of LDL and HDL were seen. LDL aggregates form some kind butterfly like features, which show great affinity towards the gold substrate because of there's hydrophilic character. Some of lipoproteins were found to be aggregated as tetrameters with a diameter of 174 nm. HDL molecules were found to be adsorbed at borders of the substrate, preferring rather substrate steps than flat terraces. The adsorbed adlayers of HDL were 38 nm high. The observed differences in the adsorbed layer structure and preferential adsorption sites were discussed from point of the different type of interactions of LDL and HDL with gold substrate. This could help to understand the arterial plaque formation helping to design nanometric scale bio-devices.

683

**Quantitative Analysis of Striated Muscle Structure by Second Harmonic Imaging**

S. V. Plotnikov,[1] A. C. Millard,[2] A. B. Isaacson,[1] V. Juneja,[1] P. J. Campagnola,[2] W. A. Mohler[1]; [1]Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT, [2]Department of Cell Biology, Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, CT
Several endogenous protein structures (e.g. collagen fibrils, myosin filaments within a sarcomere, tubulin arrays) produce strong second harmonic signals with high contrast against the background. This discrete photonic emission can be imaged at high three-dimensional spatial resolution on a laser scanning microscope yielding a detailed digital profile of the histological arrangement of native tissue samples (Campagnola et al., 2002). In this study we demonstrate two quantitative analyses of SHG relevant to muscle structure. First, we demonstrate that, as in case of collagen, myosin α-helical coiled-coil domains are the elemental harmonophores in the actomyosin lattice. Based on the assumption that SHG occurs within cylindrically symmetric arrangements of identical harmonophores, from polarization analysis we calculate the pitch angles for myosin α-helix. The pitch angle values correspond to X-ray diffraction data (Beck and Brodsky, 1998). Similarly, the angle derived for collagen agrees well with ultrastrucutral findings for the glycine-proline helix. Furthermore, polarization anisotropy of myosin and collagen SHG are significantly different allowing us to distinguish these two proteins spectroscopically. Second, we find that quantitative analysis of the periodicity, the intensity, and the regularity of the sarcomeric repeat patterns of striated muscle probed by SHG imaging may be useful in differentiating normal and diseased muscle tissue. Using hindlimb-suspended mice as a model of muscle atrophy, we observe both significant reduction in fiber size (cross section area), and areas with myosin filament disorganization and partial or complete loss of myofibril integrity. These results suggest the use of SHG imaging as a powerful tool for the structural analysis of muscle tissue and the monitoring of specific muscular disorders.

684

**Cell-Cycle Dependence of the FTIR Micro-spectroscopic Spectra of Single Proliferating Eukaryotic Cells**

S. Boydston-White,[1] T. Chernenko,[1] A. Regina,[1] M. Diem[1,2]; [1]Chemistry and Biochemistry, Hunter College of the City University of New York, New York, NY, [2]PhD Program in Chemistry, Graduate School and University Center of the City University of New York, New York, NY
We are studying the cell-cycle dependence of the FTIR and Raman micro-spectra of single proliferating eukaryotic cells to determine the spectral patterns of individual cells as a function of their state of maturation, differentiation and development. FTIR micro-spectroscopy of normal *vs.* neoplastic cells and tissues have shown differences in the spectral absorption intensities and band-shapes, particularly in the low frequency (900-1200 $cm^{-1}$) spectral region. To understand these differences, we must associate the observed spectral changes with the drastic biochemical and morphological changes occurring as a consequence of cell proliferation. We avoided any chemical perturbations leading to cell-cycle arrest in efforts to synchronize cells, but instead employed a "mitotic shake-off" to select cells in mitosis and followed them through a time course. To confirm each cell's biochemical age, we used immunocytochemical staining to detect specific temporal cellular events --- including the appearance (and subsequent disappearance) of cyclins E and B1 and the incorporation of 5-bromodeoxyuridine into nascent DNA during synthesis. Once the biochemical age was confirmed, the spectra collected could be unambiguously-assigned to particular cell-cycle phases and the spectral differences analyzed. These differences at the various time points of the cell cycle manifested themselves in pronounced changes, especially in the second derivative analysis. Shifts in the amide I peak position, indicative of different protein structures were found in the spectra of the nucleus at different time points; the amide II peak showed a distinct low frequency shoulder in the second derivative that is barely evident in the absorption spectra; and the peak at 1238 $cm^{-1}$ is observed strongly in the average second derivative spectra of all populations, but is particularly strong in the standard deviation S-phase spectrum.

685

**An Optically driven, Bacterial Screw of Archimedes**

J. R. Robbins,[1] D. Tierney,[2] H. Schmitzer[2]; [1]Biology, St. Mary's College of California, Moraga, CA, [2]Physics, Xavier University, Cincinnati, OH
The ability to rotate micromechanical elements with light is an important development in micromechanics. The three techniques to generate an opto-mechanical rotation are the spin angular momentum transfer to birefringent, micron-scale objects, the orbital angular momentum transfer by helically wound light beams and the linear momentum transfer to asymmetrically shaped structures. This final item poses the largest problem, since the production of such micro-gears is not simple. Evolution, however, provides a great diversity of engineered forms in nature. Various bacteria or cells could be useful as cheap, mass-producible elements in manmade micro-scale systems. In this work we show that the helical bacterium Rhodospirillum rubrum rotates very quickly when held in an optical tweezer. This rotation is fast enough to create a strong vortex current. It can therefore act as a micro-mixer in solution or cell culture, as well as a micro-pump or a micro-rotor to turn tiny gears in biophysical applications.

686

**Correlating Morphological and Electrophysiological Properties of Interneurons, Using Automated Image Analysis**

A. Woodruff,[1] P. Sah,[1] C. Sun,[2] L. Bischof,[2] R. Lagerstrom,[2] I. Sintorn,[2] M. Buckley,[2] P. Vallotton[2]; [1]Queensland Brain Institute, Brisbane, Australia, [2]CMIS, CSIRO, North Ryde, Australia

Electrophysiological properties of neurons allow classifying them into different subtypes based on their distinct firing properties. Interneurons in the basolateral amygdala can be distinguished into four types as shown by their different levels of adaptation in response to a prolonged (400 ms) depolarising current injection. Here, we investigate whether these observed subtypes are associated with distinct morphologies using automated image analysis of two-photon confocal microscopy data. After filling neurons with Alexa 594, we segment the measured neurites from 3D confocal stacks using a new, sensitive linear feature detector, followed by skeletonization and pruning of artefactual branches. Statistical tests based on measures of the topology of these trees revealed small differences between the different subtypes indicating that expression of different of ionic conductances, rather than morphology only, was responsible for the differences observed. This is an important finding in the endeavor towards building a mathematical model of neurons.

687

**Multiphoton Excitation-evoked Chromophore-assisted Laser Inactivation Using Green Fluorescent Protein**

T. Tanabe,[1] M. Oyamada,[1] K. Fujita,[2] P. Dai,[1] H. Tanaka,[1] T. Takamatsu[1]; [1]Pathology and Cell Regulation, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Department of Applied Physics, Graduate School of Engineering, Osaka University, Suita, Japan

Noninvasive and straightforward methods to inactivate selected proteins in the living cell with high spatiotemporal resolution are eagerly awaited for elucidation of protein function in the post-genome-mapping era. Chromophore-assisted laser inactivation (CALI) facilitates inactivation of proteins by photochemically generated reactive oxygen species (ROS), but CALI using single-photon excitation thus far has presented several drawbacks, including complex procedure, low efficiencies of inactivation with a certain chromophore, and photodamage effects. We here show that by application of multiphoton excitation to CALI using near-infrared femtosecond laser, enhanced green fluorescent protein (EGFP) can work as an effective chromophore for inactivation of a protein's function within a subfemtolitter volume without nonspecific photodamage in the living cell (MP-CALI). We found that ≈80% reductions of gap junctional currents between HeLa cell pairs expressing connexin43 (Cx43) tagged with EGFP but not between HeLa cell pairs expressing Cx43 tagged with monomeric red fluorescent protein (mRFP) were elicited by brief laser irradiation (< 500 ms) at 850-nm wavelength of the gap junctional plaque at 2.7 MW/cm$^2$ laser irradiance. Next, MP-CALI was applied to aurora B, a chromosome passenger protein, tagged with EGFP, expressed in MDCK cells. The MP-CALI parameters were as follows: laser irradiance, 1.5 MW/cm$^2$; pixel dwell time, 9.36 μs; total scan area, 10 X 13.3 μm; total scan time, 8.1 s. At metaphase, MP-CALI of aurora B-EGFP inhibited the segregation of chromosomes (14/16), which were consistent results that aurora B is required for mitosis. EGFP-mediated MP-CALI is a straightforward, non-invasive method useful for inactivation of proteins in living cells with high spatiotemporal resolution.

688

**Protein Kinase C α (PKCα) is Involved in Nuclear Factor κ B (NFκB) Signalling**

P. Morton,[1] S. Ameer-Beg,[2] M. Keppler,[1] T. Ng[1]; [1]Randall Division, Kings College London, London, United Kingdom, [2]The Advanced Technology Group, The Gray Cancer Institute, Middlesex, United Kingdom

The transcription factor NFκB is an important regulator of carcinogenesis. The Inhibitor of κB kinase (IKK) complex is essential for NFκB activation and requires phosphorylation by an unknown kinase for its activity. The aim of this study is to determine whether PKC is involved in the NFκB signalling pathway through an interaction with the IKK complex. Forster Resonance energy Transfer (FRET) analysis was performed to determine the potential interaction between IKKβ-GFP and PKCα-mRFP1 after stimulus with tumour necrosis factor α (TNFα) and Trichostatin A (TSA); two activators of NFκB signalling. Data presented shows that a time dependent interaction occurs between IKKβ-GFP and PKCα-mRFP1 after TNF or TSA treatment. Parallel assays were performed to determine the effect of inhibiting PKCα with a conventional PKC inhibitor on TNFα and TSA induced NFκB dependent luciferase activity. Results presented show that TSA induced NFκB activity was dramatically reduced by the c-PKC inhibitor, however TNFα induced activity is not affected by inhibition of c-PKC. This indicates that TSA and TNFα activate NFκB via different upstream kinases. In conclusion PKCα interacts with IKKβ after TNFα or TSA treatment, however the kinase activity of PKCα is only required for TSA induced NFκB activity. It is possible that in the TNFα induced pathway PKCα is acting as a scaffold protein for a second kinase. This study will provide insight into the aberration of NFκB signalling seen in cancer cells and may help in the design of chemotherapeutic drugs for the many cancers where NFκB is either constitutively active or overexpressed.

689

**New Reagents and Probes for Optical Switching of Biomolecules**

T. Sakata,[1] Y. Yan,[2] G. Marriott[1]; [1]Physiology, University of Wisconsin-Madison, Madison, WI, [2]Mechanical Engineering, University of Hawai`i, Honolulu, HI

We introduced a family of thiol reactive spirobenzopyrans (BIPS) and a spironaphthoxazine (NISO) as a new approach for optical switching of biomolecular interactions [Sakata et al, (2005) PNAS, 102(13), 4759-4764; Sakata et al, (2005) J. Org. Chem., 70(6), 2009-2013]. BIPS and NISO undergo efficient, rapid and reversible, optically-driven transitions between a colorless and weakly polarized spiro (SP) state and a colored, highly polarized and weakly fluorescent merocyanine (MC) state without the release of photoproducts. In this presentation, we describe the design, synthesis and characterization of new functionalized BIPS and NISO probes and reagents that can serve: (a), as photochromic acceptor probes for FRET (PC-FRET) on proteins and within membranes; (b), as part of a new approach to perturb structure and activity of protein complexes and other polymers via optical switching between the SP and MC states. The new optical switch probes were synthesized by coupling appropriately functionalized indoline derivatives with salycilaldehydes (BIPS) or nitrosonaphthol (NISO) derivatives. Spectroscopic and biochemical analyses showed that these probes can be directed to specific sites where they undergo rapid and reversible optical transitions between the SP and MC states. Suitable examples will demonstrate the potential of these probes and optical switching to study complex biological systems.

690

**Development of a Multi-well, Total Internal Reflection Microscopy System for Kinetic Screening of Plasma Membrane Translocation Events in Thousands of Single Cells in Parallel**

S. Devasenathipathy, K. Li, T. Meyer, M. N. Teruel; Molecular Pharmacology, Stanford University, Stanford, CA

We have developed a multi-well, multi-channel, wide-field, total internal reflection (TIRF) microscopy system (Teruel and Meyer, Science, 295: 1910-1912, 2002) with accurate temperature/CO2 control and rapid fluid exchange, capable of measuring up to three plasma membrane signaling parameters such as PIP3 levels and secretion events in parallel in thousands of individual cells over time. Being able to acquire large numbers of single-cell responses allows us for the first time to ask questions about the timing between plasma membrane protein activation steps and the signaling processes that control them without washing out the critical delay and amplitude information. The multi-well format was custom-fabricated by spin-coating an amorphous fluoropolymer (Cytop, refractive index: 1.34) onto a glass slide and etching a 12-well pattern into the teflon-like film using oxygen plasma etching and conventional photolithography. Each well is approximately 1.5mm in diameter in order to hold 200-300 cells. The automated fluid exchange/delivery system can exchange stimuli to cells in the entire 12-well imaging area on the order of 1 second. The cells in each of the wells are transfected with a different combination of DNA or siRNA, making it possible to carry out 12 separate translocation timecourse experiments in parallel. Using this format, we have begun screening a library of siRNAs to identify proteins that control the plasma membrane translocation of signaling reporters such as C2-domains, glucose transporter 4 (GLUT4) and the PH-domain from AKT (Tengholm and Meyer, Current Biology, 12: 1871-1876, 2002).

691

**Correlated Light and Electron Microscopic Imaging of Multiple Endogenous Proteins Using Quantum Dots**

B. N. G. Giepmans, T. J. Deerinck, B. L. Smarr, Y. Z. Jones, M. H. Ellisman; Department of Neurosciences, The National Center of Microscopy and Imaging Research (NCMIR) at UCSD, La Jolla, CA

The importance of locating proteins in their context within cells has been heightened recently by the accomplishments in structural and systems biology. While light microscopy is extensively employed for mapping protein localization, many studies require the additional resolution of the electron microscope. Here we report the application of small nanocrystals (Quantum dots) to specifically and efficiently label multiple distinct endogenous proteins for light and electron microscopy analysis. Quantum dots are both fluorescent and electron dense facilitating their use for correlated microscopic analysis. Furthermore, Quantum dots can be discriminated optically by their emission wavelength and physically by size, making them invaluable for multi-labeling analysis. We developed pre-embedding labeling criteria using Quantum dots that are straightforward and allow optimization at the light level, before continuing with electron microscopy preparation and analysis. Experiments varying fixation strength, detergent time and type are most often required to balance accessibility, preservation and retention of antigenicity. Each tissue, cell, antigen an target location presents a little different challenge. Benefits of using Quantum dots include optimization of staining procedures with examination at the LM level, straightforward multi-protein labeling at the LM and the EM, and increased penetration as compared to traditional immunogold. We show examples of double and triple immunolabeling using light, electron and correlated microscopy in cells and tissue. We conclude that Quantum dots may aid in higher-throughput analysis of determination of precise protein localization at the LM and EM levels. Supported by NIH-RR04050, NIH-NS27177, NIH-1P20-GM72033

692

**Automated Motion Detection and Quantitation of Cell Components: Application to Dynamic Populations of Microtubules in Living Cells**

A. Altinok,[1] A. J. Peck,[2] S. C. Feinstein,[3] L. Wilson,[2] K. Rose,[4] B. S. Manjunath[1]; [1]Electrical and Computer Engineering, University of California at Santa Barbara, Santa Barbara, CA, [2]Molecular, Cellular and Developmental Biology, University of California at Santa Barbara, Santa Barbara, CA, [3]Neuroscience Research Institute, University of California at Santa Barbara, Santa Barbara, CA, [4]Center for Bio-Imaging, University of California at Santa Barbara, Santa Barbara, CA

Live cell imaging technologies have advanced enormously over the past decade. However, in most cases, analysis remains a painstaking, tedious and largely manual task, limiting the quantity and quality of resulting data. Here, we describe (i) the development of novel software that automatically detects and quantifies changes in the location of objects of interest in living cells and (ii) the application of this software to the analysis of populations of GFP-labeled microtubules (MTs). More specifically, we have developed software that uses thresholding, difference analysis and lateral motion removal to automatically track and quantify the growing and shortening behavior of populations of MTs in living cells. Our new software improves dramatically upon the currently available means to analyze MT behavior. Through automated detection of all visible MTs in a fluorescence image stack in a visible cellular region (>100 MTs), each stack produces considerably more usable data in considerably less time while removing possible unintentional operator bias. Additionally, our technique preserves intact, ordered event histories of MT populations, possibly elucidating novel MT population behaviors which cannot be described by single event frequencies and average rates calculated from parsed data sets. Finally, global analysis of MT populations could reveal regional, behavioral specificities and MT population inter-relations, possibly integral to specialized processes such as cell division and neuronal outgrowth. Taken together, this highly exportable technique improves our ability to address existing questions while making it possible to use statistics to ask novel questions regarding the behavior of MT populations (and other objects of interest) that have not been approachable previously. (Supported by NSF Information Technology Research Grant 0331697.)

693

**LAMP, a New Fluorescence Imaging Assay Employing a New Class of Photoactivatible Fluorophores, Sheds Light on the Regulation of Gap Junctional Communication**

K. A. Dakin, W. Li; Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX

Gap junction channels allow adjacent cells to communicate via the transfer of small molecules and ions and play important roles in cell homeostasis and synchronization. The regulation of cell coupling through connexin channels has been under intensive investigations. We have recently developed a new fluorescence imaging technique, LAMP (Localized Activation of Molecular Probes), for studying dynamic junctional communication in fully intact cells. Using the LAMP assay, we discovered that calcium influx through store operated channels strongly inhibits gap junction channels composed of Cx43. To understand the mechanism of inhibition, we investigated the involvement of cytosolic pH ($pH_i$), local

$Ca^{2+}$ rises near sites of $Ca^{2+}$ influx, and other cytosolic factors in the gating of Cx43 channels. Since we observed a modest $pH_i$ drop during calcium influx, we examined the possibility of synergism between $[Ca^{2+}]_i$ rise and $pH_i$ abatement. Reducing $pH_i$ down to 6.5 using a weak acid had little effect on cell coupling by itself, and clamping $pH_i$ to physiological levels during calcium influx did not prevent the inhibition of junctional communication. In contrast, buffering $[Ca^{2+}]_i$ with BAPTA, but not EGTA, largely suppressed cell uncoupling by $Ca^{2+}$ influx. These results suggest that localized $Ca^{2+}$ elevation, rather than cytosolic acidification, mediates the inhibition of cell coupling during $Ca^{2+}$ influx. To extend the LAMP technique to assay cell-cell communication in three dimension, we resorted to the technique of two photon uncaging and imaging. This new class of caged fluorophores possesses very high two photon excitation cross sections at wavelengths above 700 nm, and thus are ideal fluorescent probes for imaging molecular movements in physiological preparations.

694

**A Versatile Low-cost Multi-mode Laser Microsurgery Workstation**

V. Magidson, A. Khodjakov; Molecular Medicine, Wadsworth Center, Albany, NY

Intracellular manipulations on the level of the individual intracellular organelles are becoming increasingly popular among cell biologists. There is a growing demand for workstations capable of combinational laser ablation/photobleaching/photoactivation techniques. We describe a relatively inexpensive but versatile instrument that can be assembled in an average biological laboratory and is virtually maintenance-free. The system is capable of diffraction-limited laser ablation/photobleaching and low-light 4-D imaging of live cells. It utilizes two lasers: a 532-nm pulsed Nd:YAG (Diva II, Thales; 8-ns pulses) used for ablation, and a 488-nm CW laser for photobleaching. Both beams are steered to the microscope objective lens through a dedicated epi-port available on the Nikon TE-2000 inverted microscope. This design allows us to avoid moving parts (such as dichroic mirrors or filter cubes) in the beam path which greatly improves reproducibility of resolution-limited targeting for laser ablation. Further, our design permits continuous observation of the target during laser ablation. Distortion-free attenuation of the beam is achieved via a two-stage conditioning system. First, double internal reflection on a flat parallel glass window is used to decrease the pulse power approximately 500-fold. Then, a rotatable polarizer/half-wave plate is used for finer adjustments. This design also allows us to match polarization of the laser beam to the orientation of the Wollaston prism, preventing beam splitting often observed in DIC-based systems. Variable-zoom focusable beam expander (<1/8 wavelength beam distortion - Special Optics) is used to match beam diameter to the objective lens aperture. Capabilities of our system have been proven by cutting individual microtubules and microtubule bundles in a variety of cells ranging from yeast to mammals. Further, we illustrate high precision of laser targeting by ablating just one of the two centrioles positioned <500 nm from each other.

695

**Electron Tomography of Cells and Tissues - Studying the 3D Structure of Molecular Machines in their Native Cellular Environment at Molecular Resolution**

H. Palsdottir,[1] J. Remis,[1] M. Zemla,[1] K. McDonald,[2] M. Auer[1]; [1]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, [2]University of California, Berkeley, CA

It is widely accepted that proteins do not act in isolation, but are organized into molecular machines. Using electron microscope (EM) tomography imaging we can visualize the 3D structure of such molecular machines in their native cellular environment. Our studies include the mechanoelectrical transduction and adaptation machinery of hair cells, responsible for the senses of hearing and balance. In particular we will present tomographic analysis of the extracellular links in hair cell stereocilia. We will show that a model of the candidate protein cadherin 23 is consistent with densities of the basal links, but is not consistent with tip link 3D reconstructions. Instead we propose fibronectin to be part of the tip link. We also study cell-cell and cell-matrix adhesion complexes of kidney glomerular podocytes, breast cancer acini and bacterial biofilms. To ensure faithful sample preparation we employ high-pressure freezing/freeze-substitution, followed by resin-embedding and sectioning. These structural studies are complemented by the quest for novel EM labeling methods that rely on genetically encoded tags recognized either by the membrane-permeable fluorophore ReAsH (followed by photoconversion) or by Ni-NTA-Nanogold (followed by gold enhancement). In addition to tomographic 3D data, we will present preliminary results on specific tag-based EM labeling of organelle-targeted proteins. Using transgenic zebrafish it is our goal to specifically label tagged proteins and combine high-precision 3D localization of tagged proteins with 3D ultrastructure of high-pressure frozen, freeze-substituted zebrafish larvae to obtain insight into the composition and building principles of cellular machines.

696

**High Content Kinetic Imaging Screens Identify Roles of GTPase Activating Proteins in Cancer Cell Biology**

T. R. Jackson,[1] S. Fitch,[1] C. A. Robson,[2] P. Andras,[3] R. K. Martin,[1] N. A. Daniels[1]; [1]Bioimaging, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom, [2]Northern Institute for Cancer Research, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom, [3]School of Computing Science, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom

Recent developments in automated fluorescence microscopy have allowed the emergence of high content screening {1} as a powerful tool for the investigation of integrative systems biology. Imaging allows quantitative investigation of multiple biological parameters in living cells allowing investigators to monitor the results of parallel pharmacological and molecular genetic manipulation of individual signalling pathway components. We have utilised a kinetic imaging fluorescence microscopy system to investigate the requirement for Arf-GTPase Activating protein function in regulation of the cell cycle, endocytosis and the cycle. These studies support the function of β centaurins {2} as regulators of these processes in human cancer cells. The successful execution of high content screening is dependent on access to digital imaging microscopy in a controlled cell culture environment, appropriate assay design and critically on software for automated data interrogation, quantification and visualization. We have developed and/or modified a number of novel java tools including those for measurement of cell movement and cell tracking which we have implemented for use with data stored in accordance with emerging standards of the Open Microscopy Environment {3}. {1} **Image-based screening: a technology in transition** Ramm P *Current Opinion in Biotechnology* 2005, **16** (1): 41-48. {2} **β centaurins in cancer** Martin RK, Jackson TR *Biochem Soc Trans* 2005, *in press* {3} **The Open Microscopy Environment (OME) Data Model and XML file: open tools for informatics and quantitative analysis in biological imaging** Goldberg IG, Allan C, Burel JM, Creager D, Falconi A, Hochheiser H, Johnston J, Mellen J, Sorger PK, Swedlow JR *Genome Biology* 2005, **6**(5):R47.

697

**Optimization and Use of the Biarsenical Binding Tetracysteine Motif for Fluorescence and Affinity**

B. R. Martin,[1] B. N. G. Giepmans,[2] S. R. Adams,[1] R. Y. Tsien[3]; [1]Pharmacology, University of California, San Diego, La Jolla, CA, [2]Neurosciences, University of California, San Diego, La Jolla, CA, [3]Pharmacology, Chemistry and Biochemistry, and the Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA

Membrane-permeant biarsenical dyes such as FlAsH and ReAsH fluoresce upon binding to genetically encoded tetracysteine motifs expressed in living cells, yet spontaneous non-specific background staining conceals weakly expressed or dilute proteins from detection. If the affinity of the tetracysteine peptide could be increased, then stringent dithiol washes should increase the contrast between specific and nonspecific staining. Residues surrounding the tetracysteine motif were randomized and fused to GFP, then retrovirally transduced into mammalian cells and iteratively sorted by fluorescence-activated cell sorting for high FRET from GFP to ReAsH despite increasing concentrations of dithiol competitors. Selected sequences demonstrate higher fluorescence quantum yields and drastically improved dithiol resistance, culminating in a >20-fold increase in contrast. Fusion of a tetracysteine tagged CFP (FlAsH or ReAsH) or GFP (ReAsH) to a cellular protein allows biarsenical fluorescence to be monitored by FRET from the donor fluorescent protein to the nearby, genetically fused biarsenical-tetracysteine acceptor. By excluding non-specific biarsenicals from being excited, FRET mediated detection increases ReAsH contrast almost 8-fold, making it only 2-fold less than GFP. Fusion of a tetracysteine barely increases the size of the fluorescent protein, but potentially provides the extended functionalities of the biarsenical-tetracysteine system, such as fluorescent pulse-chases, photoinactivation, and EM photoconversion. The best tetracysteine sequences, e.g. FLNCCPGCCMEP, maintain their enhanced properties as fusions to either termini of GFP or directly to β-actin, and should enable detection of a much broader spectrum of cellular proteins. Additionally, the sequence YRECCPGCCMWR fused to the N-terminus of GFP precipitates upon binding ReAsH. Fusions of this tetracysteine-GFP to the N-terminus of several cellular proteins show rapid ReAsH-triggered aggregation. This aggregation is reversible upon bleaching of the ReAsH fluorophore and may lead to a new method of protein inactivation in living cells.

698

**Wet Electron Microscopy Using Quantum Dots**

W. Timp,[1] N. Watson,[2] A. Sabban,[3] O. Zik,[3] P. Matsudaira[4]; [1]Electrical Engineering, MIT, Cambridge, MA, [2]Whitehead Institute for Biomedical Research, Cambridge, MA, [3]Quantomix Corporation, Nes-Ziona, Israel, [4]Biology, MIT, Cambridge, MA

Light microscopy is the current technique of choice for high-resolution biological imaging. However, light microscopy is limited in its ultimate resolution to $0.61\lambda/NA$ or around 100nm. Achieving this ultimate resolution is nontrivial, involving careful optimization of the microscope combined with computational deconvolution. Electron microscopy may be used to obtain higher resolution images, with a possible spatial resolution on the order of 10 nm with scanning electron microscopy (SEM). However, attaining contrast on biological samples with an electron microscope is not as straightforward as it is using optical methods. Enhancing biological sample contrast requires complicated preparation techniques, including critical point drying, coating, embedding, and sectioning. Expensive equipment and extensive training are required in order to prepare a sample for normal electron microscopy using these techniques. Instead, we have used wet electron microscopy, which is essentially electron microscopy in a sealable capsule. Specifically, we were looking for information the actin cytoskeleton of IC-21 cells. The microstructure of these actin filaments, specifically, information about how they are organized may give us more insight as to the mechanism of cell motility. In order to have sufficient contrast on these samples, we have tried a variety of stains, from uranyl acetate to colloidal gold particles. The most interesting result came from using quantum dots. Quantum dots are semiconductor particles which fluoresce at wavelengths defined by their size. Quantum dots are very useful as a stain because they are visible in light microscopy as well as in electron microscopy. This allows us to do correlative microscopy on the same sample, without any further preparation or modification. The resolution benefits granted by SEM can be used to explore further information already obtained from fluorescent microscopy.

699

**Site-specific 3D Imaging of Cells and Tissues With a Dual Beam Microscope**

J. A. W. Heymann,[1] M. Hayles,[2] I. Gestmann,[2] D. Shi,[1] B. Lich,[2] S. Subramaniam[1]; [1]National Cancer Institute, Bethesda, MD, [2]FEI Company, Eindhoven, The Netherlands

Current approaches to 3D imaging at subcellular resolution using confocal microscopy and electron tomography, while powerful, are limited to relatively thin and transparent specimens. Here we report the development of a new generation of dual beam electron microscopes that are capable of site-specific imaging of the interior of cellular and tissue specimens at spatial resolutions significantly better than those currently achieved with optical microscopy. The principle of imaging is based on using a focused ion beam to create a cut at a designated site in the specimen, followed by viewing the milled surface with a scanning electron beam. Iteration of these two steps several times thus results in the generation of a series of surface maps of the specimen at regularly spaced intervals. The three-dimensional maps obtained with this sequential “slice-and-view strategy” are at resolutions as high as ~ 10 nm in-plane and ~ 100 nm in the vertical direction. We demonstrate the potential of dual beam microscopy for biological imaging using critical-point dried yeast cells imaged at room temperature, as well as frozen yeast cells and tissue specimens imaged at -140 °C. Besides the opportunities for in situ 3D imaging of cells and tissues, we demonstrate that the dual beam microscope can be used to generate thin sections as well as cylindrically shaped specimens that can be subsequently examined in a transmission electron microscope.

700

**Application of SERS Nanoparticles to Intracellular pH Measurements**

T. A. Laurence,[1] C. Talley,[2] M. Colvin,[3] T. Huser[2]; [1]Physical Biosciences Institute, Lawrence Livermore National Laboratory, Livermore, CA, [2]Chemistry and Materials Science, Lawrence Livermore National Laboratory, Livermore, CA, [3]University of California, Merced, Merced, CA

We present an alternative approach to optical probes that will ultimately allow us to measure chemical concentrations in microenvironments within cells and tissues. This approach is based on monitoring the surface-enhanced Raman scattering (SERS) response of functionalized metal nanoparticles (50-100 nm in diameter). SERS allows for the sensitive detection of changes in the state of chemical groups attached to individual nanoparticles and small clusters. Here, we present the development of a nanoscale pH meter. The pH response of these nanoprobes is tested in a cell-free medium, measuring the pH of the solution immediately surrounding the nanoparticles. Heterogeneities in the SERS signal, which can

result from the formation of small nanoparticle clusters, are characterized using SERS correlation spectroscopy and single particle/cluster SERS spectroscopy. The response of the nanoscale pH meters is tested under a wide range of conditions to approach the complex environment encountered inside living cells and to optimize probe performance. This work was performed under the auspices of the U. S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48.

701

### Intracellular Calcium Measurements In Exocrine Cells Using Tirf Microscopy

J. Won, D. I. Yule; Pharmacology and Physiology, University of Rochester, Rochester, NY

In non-excitable cells agonist-stimulated $Ca^{2+}$ signals consist of both $Ca^{2+}$ release and $Ca^{2+}$ influx. We have investigated the contribution of these processes to near membrane $Ca^{2+}$ signals in pancreatic and parotid acinar cells using Total Internal Reflection Fluorescence (TIRF) microscopy. In extracellular $Ca^{2+}$ free conditions to isolate $Ca^{2+}$ release, physiological agonist stimulation resulted in $Ca^{2+}$ oscillations in both pancreas and parotid when measured with fluo-4 (kd=345nM). These signals did not differ significantly in profile or amplitude from those measured with wide-field microscopy. These data presumably reflect the fact that $Ca^{2+}$ release in these polarized epithelia, although triggered very close to the apical plasma membrane is occurring at some distance from the area subjected to TIRF microscopy. In contrast, when $Ca^{2+}$ influx was isolated either following agonist stimulation or after depletion of intracellular pools the $Ca^{2+}$ signals evoked were more rapid and robust than measured with wide-field microscopy and often appeared to result in saturation of fluo4, but not of the low affinity dye fluo-4 FF (kd=9700nM). Robust $Ca^{2+}$influx was initiated even at physiological concentrations of agonist which result in minimal store depletion. Interestingly, $Ca^{2+}$ influx in parotid acinar cells was 4 fold greater than that initiated in pancreatic acinar cells. These data indicate that TIRF microscopy is a sensitive measurement of $Ca^{2+}$ influx and potentially provides a useful technique to study the mechanisms which underlie these processes in polarized epithelia.

## Molecular Biology (702-722)

702

### YOMICS™ : Antibody Libraries for Functional Proteomics

M. Pagani, F. Marinoni, E. Pedrazzoli, A. Bresciani, D. Cattaneo, P. Sarmientos; Molecular Biology, PRIMM srl, Milan, Italy

In the last years, numerous studies have concentrated on the elucidation of the molecular mechanisms of human pathologies showing that most diseases are the consequence of an altered expression and/or the malfunction of one or more proteins involved in the regulation of cellular processes. A substantial acceleration in the elucidation of the molecular mechanisms underlying the diseases development and in our ability to diagnose and cure maladies is expected to take place in the next few years due to the application and implementation of high-throughput technologies that stem from the scientific revolution brought about by genome sequencing and analysis (genomics and bioinformatics), that includes transcriptome analysis (transcriptomics) and proteome analysis (proteomics). The availability of a "draft" of the entire human genome sequence and, most important, the accurate annotation of the sequences of several human chromosomes virtually allows the physical isolation of all human genes Here we describe our reverse-proteomics approach, called YOMICS, applied to the analysis of several uncharacterized human transmembrane and secretal proteins. This approach has the aim to develop high-value tools to allow a rapid definition of protein expression profiles in both normal and pathological human samples. PRIMM has developed a high-throughput cloning and expression technology. Genes are amplified, cloned into the PRIMM developed vectors and the derived recombinant plasmids are used to transform different *E. coli* strains. Once expressed, the proteins are purified by affinity chromatography and used for polyclonal antibody production. The availability of a sera library against a large panel of human proteins is the key feature of the YOMICS project. We are employing these antibodies and the purified proteins for protein-protein interaction studies, differential expression and localization analysis in normal or pathological conditions using *in vivo* (eg. FACS) and in vitro (eg. immunochemistry) analysis techniques.

703

### High-Throughput and High-Yield Protein Expression

T. W. Sproul, A. M. Burt, R. D. Guettler, L. T. Mazzola; Excellin Life Sciences, Menlo Park, CA

Expression cloning is an important tool in academic and industrial research, whether for transient expression of cDNA libraries or stable incorporation for production clones. Recently there has been a push toward high-throughput expression analysis; however, this approach lacks a consistent front-end process for high-throughput generation of transfected cells. Current methods for transfection can be extremely labor intensive and can't be used for all cell types. Furthermore, high levels of cDNA induction are often desirable to differentiate exogenous vs. endogenous expression - current transfection methods limit the amount of material that can be safely delivered into the cells. Here we demonstrate high levels of expression easily and reliably achieved for cytosolic, transmembrane and secreted proteins using a novel microelectronic transfection process. Transfection achieved 80-95% overall efficiency (90-95% cell viability), as indicated by dsRed, GFP, PAG-GFP fusion and CD4 protein production in a variety of cell lines. This technique is consistent with high-throughput and high-volume processing for both suspension and *in situ* adherent cell types - including primary and stem cells.

704

### Novel Nanoelectronic Chips for Gene Profiling and Proteomics

H. Chen, A. M. Cassell, J. Koehne, M. Meyyappan, J. Li; Ames Research Center, Moffett Field, CA

Microarray is an important tool for high throughput analysis of biomolecules. The use of microarrays for parallel screening of nucleic acid and protein profiles has become an industry standard for drug discovery and biomarker identification recently. But it requires relatively large sample volumes and elongated incubation time due to the large spot size. This also limits the sensitivity of detection. In addition, traditional microarray uses bulky instrument for the detection requires major efforts in sample amplification and labeling, which increase the cost and delay the time for getting the results. These drawbacks are the biggest impediment to move the nucleic acid and protein based detection to point-of-care and field applications. To overcome above limitations, Carbon Nanotube (CNT) based nanoarrays are developed by using nanotechnologies. In this nanoelectrode array platform, individually addressed ultra-microspot is about 10 μm x 10μm$^2$, consisting of tens or hundreds of nanoelectrode with

a diameter of 50 to 100 nm, which are inlaid in insulating materials such as $SiO_2$ with only the very end exposed. We have demonstrated them for ultrasensitive nucleic acid analysis by label-free electrochemical detection. These nanoarrays are also being developed for highly sensitive protein filing. CNT nanoelectrode arrays are also much easier in automation and date analysis. These nanoarrays will have significant applications in drug discovery, medical diagnosis, genetic testing, environmental monitoring, and food safety inspection with novel gene profiling and proteomics tools

705

**Two-Dimensional Organelle and Organellar Subtype Fractionation for Subcellular Proteomics Studies**

H. Tran, D. E. Kalman, J. A. Smith, W. Lan; Alfa Wassermann Proteomic Technologies, LLC, West Caldwell, NJ

The challenge of subcellular proteomics lies with the separation, fractionation and enrichment of organelles and organellar subtypes. Here we present a 2-dimensional centrifugation technique: our novel proteomic continuous-flow ultracentrifugation (pCFU$^{TM}$) combined with differential centrifugation to achieve the dynamic range of organelle fractionation. Initially, pCFU separates and enriches organelles at their buoyant density using a sucrose gradient. The continuous-flow nature of the technique allows accumulation of the organelles due to the unlimited amount of sample that can be loaded. Differential centrifugation of pCFU fractions further separates organelles by their sedimentation rates. In this study, HeLa cells were homogenized and nuclei were removed by low speed centrifugation. After 2-dimensional ultracentrifugation, Western blot analysis showed significant differences in organelle distributions between 2-dimensional fractionation and differential centrifugation alone. The differences in the mitochondria patterns between fractions from pCFU demonstrated the power of separation of organellar subtypes. Furthermore, compared to the starting material the fractions from pCFU showed enrichment and accumulation of organelles. Fractions from the 2-dimensional organelle fractionation process were subjected to SDS gel electrophoresis for separation of proteins. Slices from these gels were then analyzed by nano-LC-ESI-MS/MS for protein identification. With 65 fractions from pCFU, 4 fractions from differential centrifugation and 20 slices from SDS gel electrophoresis, a potential of 5200 samples can be analyzed by mass spectrometry which provides the ability to analyze the entire proteome.

706

**Mapping and Functional Characterization of Phosphorylation Sites in *Drosophila* Flightin**

D. J. Lemas,[1] M. Previs,[2] J. Vigoreaux[1]; [1]Biology, University of Vermont, Burlington, VT, [2]Cell and Molecular Biology, University of Vermont, Burlington, VT

Flightin is a novel 20-kD myofibrillar protein found in *Drosophila* indirect flight muscles (IFM), the principal muscles that power flight. Previous studies have shown that flightin is essential for normal thick assembly during muscle development and required for thick filament stability in active, adult contracting muscle. Evidence suggest that phosphorylation of flightin plays a dual role in dictating thick filament length during development and modulating the contractile properties of the muscle. Currently eleven flightin isoelectric variants, two non-phosphorylated variants (referred to as N1 and N2) and nine phosphorylated variants (P1 through P9), have been identified in vivo by two-dimensional gel electrophoresis (2DE) of IFM fibers. We are conducting matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of 2DE gel spots to identify the phosphorylation sites in flightin and to determine if each 2DE spot constitute a single form or a mixture of different phosphorylated forms. Phosphorylation site mapping shows that most sites are found in clusters in the C-terminal two thirds of the protein. Phylogenetic analysis revealed that some of the sites are not conserved among *Drosophila* species suggesting that their role may be modulatory. To test the hypothesis that phosphorylation of flightin is a mechanism for modulating power output, we are measuring the relative levels of flightin isovariants in non-flying and flying *Drosophila melanogaster* by quantitative 2DE. Our results show dynamics changes in the relative abundance of non-phosphorylated and phosphorylated flightin isovariants as flies transition from rest to full flight. This study will provide new insight into the role of phosphorylation in dictating the contractile dynamics of muscle *in vivo*.

707

**Behavior of Protein Complexes in Native Two-Dimensional Gel Electrophoresis**

J. E. Smotrys, L. Chen, C. S. Giometti; Argonne National Laboratory, Argonne, IL

Protein complexes play a major role in the normal metabolism of all cell systems. Methods for the isolation of intact complexes continue to be a challenge, since the association constants between the components vary. Two-dimensional (2D) gel electrophoresis is a powerful tool for the separation of complex protein mixtures and provides the opportunity to identify the proteins when coupled with peptide mass analysis. Electrophoresis of proteins under nondenaturing conditions preserves their native conformations, allowing for detection of enzymatic activities and the identification of the components comprising protein complexes (Giometti *et al*. 2003 *Proteomics* 3:777). Two questions regarding native gel electrophoresis are addressed in this work. First, do known protein complexes remain associated during 2D native electrophoresis? We have demonstrated that trypsin and soybean trypsin inhibitor form a complex that survives the high voltage conditions experienced during 2D native electrophoresis. Secondly, what is the size limitation of gel separation-based methods? Native protein samples are likely to contain very high molecular weight complexes that may be excluded from entry into traditional polyacrylamide gels because of their size. Using proteins with a range of molecular weights, we have shown that a mixture of agarose and acrylamide can be successfully utilized for second-dimension electrophoresis that allows greater gel penetration of high molecular weight proteins than use of polyacrylamide alone. These results confirm the important role that 2D electrophoresis can play in future proteomic studies that emphasize protein functions and interactions.

708

**Developing an Add and See Array System Using FRET(Fluorescence Resonance Energy Transfer) to Detect Protein Interaction**

J. Lu, P. C. Byrne; Pathology, UCD, Dublin, Ireland

The problems associated with developing high-throughput screening of protein interactions on a protein chip include difficulties in determining whether (i) the proteins on the chip are denatured, (ii) the specificity of the reaction and (iii) the relative strength of protein interaction. The multiple steps required in most current systems hamper widespread use and commercialisation. Here, We provide preliminary results on the development of an add and see (A&C) array system using FRET (Fluorescence Resonance Energy Transfer) to detect protein interaction. A library of prokaryotic and eukaryotic plasmids expressing enhanced fluorescent protein tagged (EFP, i.e. EYFP/ECFP) target or probe fusion protein sequences is prepared. The plasmids with EFP-probe and EFP-target fusion proteins also have different antibiotic resistance genes. Both plasmids

were heat-shock co-transfected into E coli. Protein expression was induced by addition of IPTG. The E. coli expressing target proteins were loaded onto multi-well plates or slides (array/chip, Add). The cells in the array were fixed and scanned for FRET reaction to indicate the existence of protein interaction (See). Array cells that were positive for FRET are further examined in a eukaryotic system. The method is easy to standardize for automation. With EFPs used as quality and quantity internal controls, it is possible to perform intra- and inter-chip checks and measure the relative binding strength by calculating the FRET efficiency. We are looking for collaborators and sponsors for proteomic scale work with this system.

709

**Blotless Nano Western - A New Approach for High Performance Protein Assays of Stem Cells and Other Precious Biological Materials**

E. T. Gentalen, X. Bi, J. Ferrante, J. Gossett, C. Kirby, B. Loder, M. MacLaren, T. Mills, R. O'Neill, N. Parker, D. Roach, D. Rubin, D. Suich, P. VanderHorn, D. Voehringer, K. Voss, T. Yang; Systems Integration, Cell Biosciences, Palo Alto, CA

The ability to assay low abundance proteins in stem cells and other precious biological materials (primary cell lines, repeat sampling in animal models, tissue microdissections, etc.) is a critical need of life science research. In spite of a proliferation of new methods, the arduous Western blotting technique remains the most commonly utilized protein assay method. Using the fundamental strengths of Western blotting - the combination of a physical separation with antibody binding and signal amplification - we have developed a high performance, automated nano-volume immunoassay platform. Advantages of this system include high sensitivity (e.g. < 1000 stem cells), quantitative analysis and full automation. Additionally, the system performs separation and single antibody detection of phosphorylated and non-phosphorylated protein isoforms enabling insights into cell signaling previously unachieved. To meet these goals, a capillary electrophoresis platform incorporating a unique concept of locking proteins in place following a charge-based separation was developed. After protein separation and capture, antibodies are flowed through the capillary where they bind to their target proteins. In the standard method, primary antibodies are then bound by secondary antibodies with attached chemiluminescence reporter enzymes. Finally, whole capillary images are captured while chemiluminescent substrate is flowed through the capillary providing continuous luminescence detection without exhaustion of substrate. The system processes multiple capillaries in parallel similar to multiple lanes of a traditional Western analysis. Automation allows the unattended analysis of a full 96-well plate of samples in approximately 4 hours. The platform design is fundamentally scaleable to extremely high throughput capability similar to the evolution of modern DNA sequencers.

710

**Over a Hundredfold Increase in Immunoblot Signals of Laser-microdissected Inclusion Bodies with an Excessive Aggregation Property by Oligomeric Aip2p/Dld2p**

N. S. Hachiya,[1] T. Ohkubo,[2] Y. Kozuka,[3] Y. Sakasegawa,[4] K. Kaneko[1]; [1]Second Department of Physiology, Tokyo Medical University, Tokyo, Japan, [2]Department of Neurology and Neurological Science, Tokyo Medical and Dental University, Tokyo, Japan, [3]National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan, [4]Department of Prion Protein Research, Tohoku University, Tokyo, Japan

By utilizing non-chemical denaturant (oligomeric Aip2p/Dld2p) with a combinatorial method of laser-microdissection and immunoblot analysis, we have established a histobiochemical apporoach targeting micron-order inclusion bodies with an extensive aggregation property *in situ*. As a model, pick bodies were chosen and laser-microdissected from 3 different brain regions of 2 patients with Pick's disease. Five hundred to 2,000 pick bodies were initially applied onto SDS-PAGE gels after boiling in Laemmli's sample buffer according to the established immunoblotting procedure, but only faint signal was obtained. Following negative results with chemical denaturants or detergent including 6 M guanidine hydrochloride, 8 M urea, and 2 % SDS, the laser-microdissected pick bodies were pretreated with oligomeric Aip2p/Dld2p, which exhibits a robust protein unfolding activity in biological condition. Strikingly, only one pick body was sufficient to illustrate an immunoblot signal, indicating that the pretreatment with oligomeric Aip2p/Dld2p enhanced the immunoblot sensitivity over a hundredfold. The pretreatment with oligomeric Aip2p/Dld2p also allowed us to quantify the total protein contents of pick bodies. Such unprecedented property of the oligomeric Aip2p/Dld2p may imply further potential applications. For example, a number of proteomic strategies rely on liquid chromatography-tandem mass spectrometry (LC-MS/MS), but sample preparation methods typically involve the use of detergents and chaotropes that often interfere with chromatographic separation and/or electrospray ionization. The oligomeric Aip2p/Dld2p, however, does not interfere with the LC-MS/MS procedures, and it could be an ideal pretreatment. Overall, oligomeric Aip2p/Dld2p may greatly facilitate the nano-scale analyses, which are often hindered from the aggregation property of target proteins in various protein analytical procedures, especially in minor quantities.

711

**Co-Detection of Low-Abundant Proteins in Signal Transduction Pathways**

S. Edlund,[1] J. Lennartsson,[2] M. Landstrom,[2] A. Edman-Örlefors,[1] A. Hagner-McWirther,[1] A. Hyuronowitz,[1] O. Ronn[1]; [1]Bio-Sciences, GE Healthcare, Uppsala, Sweden, [2]Ludwig Institute for Cancer Research, Uppsala, Sweden

Detection of proteins using Western blotting is a well known technique, most often based on chemiluminescense. A limitation is that only one antigen at a time can be detected. A stripping procedure has to be performed on the membrane before probing for the next protein. This will result in loss of material and reduced reliability of quantitations. Studies on low-abundant proteins, e.g. phosphoproteins, depend on reliable quantitative analysis methods. For this purpose a new Western blotting technique, based on antibodies conjugated with fluorescent molecules, has been developed and optimized. This enables simultaneous detection of two proteins or protein states (e.g. phospho- versus total protein levels) on the same membrane. Together with an increased dynamic range and high sensitivity this gives more reliable quantitations and reduced variability of activated low abundant proteins, like phosphoproteins. In this study we have been able to study proteins involved in signaling pathways that regulate cell growth, survival, actin reorganization, migration, differentiation and apoptosis, in response to the extracellular proteins platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-ß). We could detect the tyrosine phosphorylation of the PDGF receptor upon PDGF-BB stimulation of porcine aortic endothelial (PAE) cells. The simultaneous protein blot detection was done using primary antibodies for the phosphotyrosine protein which could be related to the total receptor levels (PDGFR-β) by using multi-wavelength fluorescent secondary antibodies. We could also detect and make accurate quantifications of the TGF-β-mediated phosphorylation of p38 in human T293 epithelial kidney cells. Here, human T293 epithelial kidney cells were activated with TGF-β and harvested at different time points. The TGF-β-mediated

phosphorylation of p38 shows proportional increase, of pp38, when compared to actin.

712

**Improvement in Large-scale Production of PCR-generated Small DNA Fragments for a Gene Therapy Approach**

D. de Semir; CPMC, San Francisco, CA

Background: One novel gene therapy repair strategy, Small Fragment Homologous Replacement (SFHR), uses small DNA fragments (SDFs) generated by PCR and has demonstrated its effectiveness in altering gene sequences both *in vitro* and *in vivo*. A necessary factor to improve the efficiency of SFHR-mediated gene modification requires the establishment of a protocol for the production of large-scale SDF of good quality. Objective: Validation of a method to produce large quantities of SDFs not exposed to UV light nor to ethidium bromide (EtBr) for electroporation into two human lymphoblastoid cell lines containing a point mutation on exon 5 and 6 of the interleukin-2 receptor common gamma chain gene (*IL2RG*). Methods**:** Various SDFs differing in size including the wild-type *IL2RG* gene sequence were amplified by PCR from genomic DNA isolated from a non-SCID cell line and cloned. The clones sequenced were used as a template for large-scale generation of SDFs containing the wild-type sequence. The pooled DNA fragment was ethanol precipitated, quantified spectrophotometrically, and purified by gel extraction. However, only the lane containing a small sample of the pooled DNA was stained with EtBr and visualized by UV. The remaining pooled DNA fragment was sliced out of the gel based on the position of this visualized reference sample. Studies are now underway to evaluate the efficacy of the transfected SDFs. Results**:** a large amount of SDFs not exposed to EtBr nor to UV can be obtained by gel extraction purification. Conclusions: the non use of UV nor EtBr increases the purity and quality of the PCR-generated SDF for gene therapy approaches as it doesn´t contain possible T-T adducts created by UV nor intercalating agent in its sequence, then reducing its toxicity when transfected into target cells.

713

**Targeted Disruption of Transcriptional Regulatory Function of p53 by a Novel Efficient Method for Introducing a Decoy Oligonucleotide into Nuclei**

T. Nukui,[1] M. Sakaguchi,[1] H. Sonegawa,[1] H. Murata,[2] J. Futami,[2] H. Yamada,[2] N. Huh[1]; [1]Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikatachou, Okayama, Japan, [2]Department of Bioscience and Biotechnology, Okayama University Graduate School of Natural Science and Technology, Tsushimanaka, Okayama, Japan

Decoy oligonucleotides have been used for functional sequestering of transcription factors. Efficient introduction into cells is a prerequisite for the oligonucleotides to exert their blocking function. Lipofection is the most widely used technique for that purpose because of its convenience and relatively high efficiency. However, the transduction efficiency of lipofection largely depends on cell-types and experimental conditions and the introduced nucleotides are not specifically directed to nuclei where they exert their major function. In the present study, we designed a new system for transporting oligonucleotides into cell nuclei. The vehicle is composed of glutathione-S-transferase, 7 arginine residues, the DNA-binding domain of GAL4, and a nuclear localization signal, which are linked with flexible glycine stretches. The p53 responsive element linked to the GAL4 upstream activating sequence was efficiently transferred by the vehicle protein into nuclei of primary cultures of neuronal cells, ES cells and various human normal cells. Transcriptional activation of $p21^{WAF1/CIP1}$ and Bax by p53 on exposure to cisplatin was completely blocked by introducing the p53 decoy oligonucleotide. Thus, the system developed in the present study can be a convenient and powerful tool for specifically disrupting the function of DNA binding proteins in culture.

714

**Adenoviral-mediated Transgene Expression Enhanced by Etoposide Co-treatment in Cultured Cells**

J. W. Triplett, B. P. Herring, F. M. Pavalko; Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN

The overexpression of proteins in cell culture systems is an effective way to assess the function of proteins in cells. In recent years, the development of adenoviral-mediated gene transfer systems has enhanced the ability of researchers to express exogenous proteins to a high level in multiple cell types. However, expression in certain cell types and expression of certain constructs remains difficult even with adenoviral-mediated gene transfer. We investigated the potential of the topoisomerase inhibitor etoposide to increase adenoviral-mediated gene expression in cell culture systems. Previous studies indicate that the use of DNA-damaging agents, such as topoisomerase inhibitors, may increase adeno-associated viral-mediated transgene expression both *in vivo* and *in vitro*. Titration of etoposide in the presence of equal volumes of adenovirus demonstrated that treatment with as little as 100 nM etoposide for the first 24 hours of infection increases transgene expression up to 3-fold in both UMR106 and REF52 cells. Importantly, subsequent studies indicated that low doses of etoposide did not cause cytotoxicity, as measured by lactate dehydrogenase release, nor apoptosis, as measured by western blotting for apoptotic markers. Additionally, equivalent transgene expression was achieved using approximately five-fold less volume of virus. Finally, the use of etoposide did not interfere with the ability of exogenous proteins to function normally within cells, as evidenced by the ability of myocardin to increase smooth muscle-specific protein expression in osteosarcoma-like cells even in the presence of etoposide. Together these data indicate that etoposide may be an effective means to enhance the expression of adenoviral constructs in several cell types without inducing cytotoxic nor apoptotic effects. The use of etoposide may be an inexpensive way to both increase adenoviral transgene expression in cultured cell systems and prolong the life of adenoviral stocks.

715

**Generation of a Cre/LoxP-based Conditional hCXCR1 Transgenic Mouse Line with Color-Switching Indicators to Study the Functions of hIL-8 *in vivo***

L. Zheng, M. Martins-Green; Department of Cell Biology and Neuroscience, University of California, Riverside, CA

The CXC chemokine Interleukin-8 (IL-8) plays pivotal roles in inflammation, wound healing and angiogenesis. Human IL-8 has two 7-transmembrane G-protein-coupled-receptors, CXCR1 and CXCR2, both of which mediate the functions of IL-8 in human but in different ways. We used a Cre/LoxP-based transgenic vector to introduce hCXCR1 into mice, which enables controlled expression of hCXCR1 in both location and time. The C-terminus of hCXCR1-ORF was fused to the tetracysteine (C4) motif, which serves as binding motif for biarsenical fluorescent reagents for *in situ* labeling of recombinant protein in live cells. A fusion protein of firefly luciferase and eGFP (Luc-eGFP) was designed to target onto the plasma membrane with eGFP on the outside and the luciferase moiety inside. This will facilitate tracing of cells *in vivo* and cell separation

*in vitro*. The ORFs for hCXCR1-C4 and Luc-eGFP were incorporated into an IRES-based bicistronic transcript driven by a potent and ubiquitous promoter that is active in all mouse tissues. A floxed monomeric red fluorescent protein (mRFP) ORF+3xPolyA was inserted between the promoter and the bicistronic transcript to render the hCXCR1 expression silent in the F1 founder mice. When these mice are crossed with Cre-recombinase expressing mice, the mRFP1+3xPolyA is deleted and the hCXCR1-C4 and Luc-eGFP expression are activated. Tissue-specific and other cre-mice will facilitate various ways of hCXCR1 targeting by crossing with the F1 founder mice. This transgenic hCXCR1 mouse line can be a useful system to study IL-8's function *in vivo*, and the transgenic vector used here can also be a generic transgenic vector to deliver other genes of interest, providing several useful features for biological manipulations and imaging.

716

**Bacteriophage Recombinase Stimulates Single-strand DNA Oligonucleotide-mediated Gene Targeting in Human Cells**

K. Xu,[1] A. Stewart,[2] A. C. Porter[1]; [1]Department of Hematology, Imperial College London, London, United Kingdom, [2]Genomics, University of Technology, Dresden, Germany

Gene targeting uses homologous recombination (HR) to create defined changes in cellular DNA with great specificity and flexibility. This can be achieved by use of double-stranded DNA (dsDNA) gene targeting vectors or single-strand DNA oligonucleotides (ssODN) to introduce the desired changes. The efficiency of these processes is low, and improvements are required if genome modification is to be fully exploited as a tool in cell biology or as a form of gene correction therapy. In *Escherichia coli*, bacteriophage recombination systems (Redα/Redβ or RecE/RecT) have been used successfully to modify both plasmid and chromosomal DNA in a highly efficient manner, using either a linear dsDNA fragment or a ssODN. In this study, we have established human HT1080 cell lines with tightly regulated Redβ expression, and Redβ localized in nuclei. We find that Redβ stimulates homologous recombination between co-transfected plasmids and both dsDNA- and ssODN-mediated gene targeting ~2-fold. We also show that transient depletion of the mismatch repair protein MSH2, by RNA interference, stimulates ssODN-mediated episomal gene targeting ~2-3-fold. We show further that Redβ expression and MSH2 depletion can be combined to stimulate episomal gene targeting ~ 6-fold. These studies therefore describe new ways to improve gene targeting efficiencies. We are currently investigating whether the effects of Redβ expression can be stimulated by co-expression of Redα. Future optimisation and combination of these and related approaches may facilitate the generation of mutant cell lines and the therapeutic repair of mutated disease genes.

717

**Design of a Quantitative Method for Detection of Allele-specific RNA Expression**

E. D. Klootwijk, P. J. Savelkoul, C. Ciccone, D. M. Krasnewich, W. A. Gahl, M. Huizing; NHGRI, NIH, Bethesda, MD

Hereditary Inclusion Body Myopathy (HIBM) and sialuria are two distinct disorders resulting from mutations in the same gene, *GNE*, coding for the bifunctional, rate-limiting enzyme in sialic acid biosynthesis, i.e., UDP-GlcNAc 2-epimerase (GNE)/ManNAc kinase (MNK). Sialuria is caused by dominant mutations in the allosteric site of GNE/MNK, leading to a loss of feedback-inhibition and increased sialic acid excretion. In contrast, HIBM is caused by recessive *GNE* mutations outside the allosteric site, resulting in decreased GNE/MNK enzyme activity and decreased sialic acid production. Both sialuria and HIBM exhibit variable clinical phenotypes. We examined if mutation-dependent variations in allelic expression of *GNE* could account for the variable disease phenotypes. We developed a real-time RT-PCR method that rapidly and accurately detects and quantifies allele-specific expression. The procedure is based on the use of a combination of two allele-specific fluorescent reporter probes and real-time amplification kinetics. We first tested the validity of each allele-specific assay across a concentration range obtained by mixing cell-free transcribed normal and mutated *GNE* RNA. Each of the assays proved to be accurate and mutation-specific, allowing us to study allelic expression of *GNE*. We applied the assays to RNA obtained from fibroblasts of sialuria or HIBM patients. No patient showed a significant difference in mutation-dependent allelic *GNE* expression, indicating that allelic expression did not cause the variable phenotypes in these patients with sialuria or HIBM. This novel, allele-specific RNA quantifying method is convenient and rapid, and requires minimal concentrations of RNA (<25ng). The procedure is attractive for various applications, including validation of SI-RNA silencing experiments. In fact, we have demonstrated the method's validity for allele-specific RNA gene silencing using SI-RNAs in sialuria fibroblasts. In addition, this method can be employed for studies of X-chromosomal inactivation, genetic imprinting, and epigenetics.

718

**Method Development and Optimization for Automated Fluorescent (Q-DOT®) In-Situ Hybridization**

A. Birmingham,[1] L. K. Samadzadeh,[1] K. A. Vanpatten,[1] K. Hopkins,[1] A. Sattler,[1] C. Bauer,[1] C. Beinarz,[1] P. J. Beresford,[1] T. M. Grogan,[1] R. B. Nagle,[2] P. Miller,[1] G. A. Pestano[1]; [1]Discovery, Ventana Medical Systems, Tucson, AZ, [2]Pathology, University of Arizona Cancer Center, Tucson, AZ

The ability to rapidly detect genetic abnormalities has become increasingly important following the sequencing of the human genome. The recognition of genetic links to human disease states in oncology clearly illustrates the need for novel diagnostic applications. Candidate diagnostic tests need not only be reproducible, but also developed with a high degree of sensitivity. In addition, the complexity of the gene-linked diseases has resulted in the need to simultaneously monitor multiple markers. The objective of this study was to apply our automated methods to the development of ISH protocols that may be rapidly delivered and conducted reproducibly, in a highly sensitive manner. A matrix of conditions was tested for developing ISH applications. The candidate test for the development matrix was to detect the epidermal growth factor receptor (EGFR) gene in formalin-fixed and paraffin-embedded (FFPE), cell lines and in tissues. EGFR gene amplification has been linked to disease progression in a number of studies. Detection was conducted in positive controls, including in head and neck cancer and in normal skin, with a chromogenic assay (CISH) and validated in a separate Quantum Dot (QDot®) application. We report a matrix of conditions that may be used to rapidly develop a new ISH procedure in less than 72 hours. The EGFR ISH protocol was highly reproducible in both normal and neoplastic tissues, sensitive to single gene-copy detection, and amenable to archiving. The disclosed method of assay development will be highly relevant for the detection of aberrant gene and protein regulation events.

720

### An ELISA Based Rho Activation Assay

L. Cheng, K. M. Middleton, A. S. Davis; Cytoskeleton Inc, Denver, CO

The Rho family of small GTPases consists of at least 20 members, the most extensively characterized of which are the RhoA, Rac1 and Cdc42 proteins. In common with other small GTPases, the Rho proteins act as molecular switches that transmit cellular signals through downstream effector proteins by alternating between active GTP-bound and inactive GDP-bound states. The Rho family mediates a wide range of cellular responses, including cytoskeletal reorganization, regulation of transcription, membrane trafficking and apoptosis. Therefore, understanding the mechanisms that regulate activation and inactivation of the GTPases is of great biological significance and is a subject of intense investigation. The fact that Rho family effector proteins will specifically recognize the GTP-bound form of the protein has been used to develop affinity purification assays to monitor Rho protein activation. Traditionally, this has been done by coupling Rho binding domains (RBD) of Rho effector proteins to agarose beads, which are used to precipitate the active Rho from a biological sample. However, this method suffers from being time-consuming, requiring large amount of sample, yielding inconsistent results and not being suitable to high throughput screens. To avoid these drawbacks, we have developed an ELISA based assay to detect Rho activation. This novel Rho activation assay uses a 96-well plate coated with RBD domain of Rho effector proteins. The active Rho, but not inactive Rho, from a biological sample will bind to the plate. Bound active Rho is detected by incubation with an anti-Rho antibody followed by a secondary antibody conjugated to HRP and chemiluminescence. This ELISA based method is simple, fast, requires only small amounts of sample and yields quantitative and consistent results. It will therefore be a powerful tool to investigate Rho activation.

721

### Study Denitrifying Bacteria in Marine Sediments by In-Situ RT-PCR

G. Toro, G. Rodriguez, E. Padilla, E. Perez, A. Massol; Biology Department, University of Puerto Rico, Mayaguez, PR

The constant development of molecular techniques for the identification of bacteria in environmental samples is essential for understanding the interactions and roles of microbes in nutrient cycling. In situ Reverse Transcription PCR (RT-PCR) has become a novel technique to study gene expression. We are applying this approach to study the expression of the nirS gene in denitrifying bacteria. Pure cultures of model denitrifying isolates from Puget Sound marine sediments were used to optimize the RT-PCR protocol and we are now applying this technique directly from environmental samples. In order to ensure the presence of active denitrifying populations, marine sediments were bioaugmentaded with Paracoccus denitrificans and biostimulated with a nitrate solution. Unamended sediments were used as reference samples. After 48 hours of incubation, cells were extracted in a Phosphate/Tween 80 solution, filtered and concentrated by centrifugation. Samples were fixed with RNase free 4% paraformaldehyde and RNAprotect™Bacteria Reagent. Subsequently, cells were enzymatically permeabilized with lyzozyme and Proteinase K and spotted on microscope slides. In situ RT-PCR was performed using the QIAGEN® OneStep RT-PCR Kit. Amplification of cDNAwas performed using the nirS 1F and nirS 6R primers followed by Fluorescent In Situ Hybridization (FISH). A Cy-3 labeled internal oligonucleotide probe designed to detect the nirS gene in Pseudomonas stutzeri was used to specifically detect the gene of interest. Slides were then examined by fluorescence microscopy. Detectable cells were obtained from all marine sediments but at higher frequencies in bioaugmentated/ biostimulated systems. The outcomes obtained provide the basis for rapid detection of specific microorganisms in environmental samples without the need of cultivation or even nucleic acid isolation in marine sediments.

722

### A Novel Multiplex-like ELISA Test Reveals that both Gla-Clotting and Anti-Calcification Proteins are Present on Calcifying Nano-Particles

K. M. Aho,[1] O. E. Kajander,[2] N. Ciftcioglu[3]; [1]Department of Biochemistry, University of Kuopio, Kuopio, Finland, [2]Nanobac Life Sciences, Tampa, FL, [3]NASA Johnson Space Center, Houston, TX

OBJECTIVE Calcifying Nano-Particles (CNP), also called Nanobacteria, are tiny particles in blood with the ability to form apatite on them. Our studies on biodistribution of CNP revealed that colloidal CNP caused arterial and venous thrombosis in a significant number of rabbits and rats within minutes after i.v. administration. Our aim was to determine why CNP enchance blood clotting/thrombosis. A novel Multiplex ELISA was developed to probe with specific antibodies the possible presence of Gla-proteins, having high binding affinity towards apatite, on CNP. METHODS CNP were detected with quantitative Nanocapture ELISA kit (Nanobac Oy, Finland). The capture antibody was replaced with several specific anti-Gla-protein antibodies, including antibodies for blood clotting factors FII, FVII, FIX and FX/Xa and blood anti-mineralization proteins. The same detection antibody from the Nanocapture kit was used in all tests and plasma and serum gave comparable results. 16 pooled human serum samples (2 negative and 14 positive representing CNP values from 0 to 640 Capture units) were assayed with all capture antibodies. Correlation values between Nanocapture and various Multiplex ELISA variants were determined with Pearson Correlation Coefficients. RESULTS Pearson Correlation Coefficients indicated presence of all clotting Gla-proteins and matrix-Gla protein in CNP. The pattern of their presence correlated with Capture unit results for CNP ($p < 0.001$ for all of them). CONCLUSIONS Multiplex ELISA proves that the human blood CNP contain FII, FVII, FIX, FX/Xa and matrix-Gla protein. Likely binding mechanism to CNP is the high affinity of their multiple Gla-residues to apatite. Thus CNP particles have their own coagulation system in addition to anti-calcification system. The Multiplex ELISA is suitable for detecting the Gla-proteins and other proteins on CNP, and provides a novel type of assay for analyzing CNP pathological proteins.

## Blood Vessels (723-733)

723

### Sphingosine 1-phosphate Signaling in the Vascular System

T. Hla; Center for Vascular Biology, Univ of Connecticut Health Center, Farmington, CT

Metabolism of sphingomyelin, an abundant membrane phospholipid by the sphingomyelinase pathway results in the formation of sphingosine 1-phosphate (S1P), a pleiotropic lipid mediator. Our laboratory has shown that S1P acts via the G protein-coupled receptors of the EDG family, which are renamed as S1P1-5 receptors. S1P receptors are present in vertebrates and regulate vascular development, angiogenesis and immune cell

trafficking. In mammals high plasma S1P and low tissue S1P levels establish a vascular S1P gradient. The cellular basis of this gradient is not clearly understood. Sphingosine kinase-1a isoform is secreted from vascular endothelial cells and contribute to the high plasma S1P levels. Different sphingosine kinase isoforms are localized at different subcellular locales and produce S1P, suggesting that site-specific formation of S1P is critical for various biological activities of this lipid mediator. In the vascular system, S1P1 receptor activates the PI-3-kinase/ Akt pathway to regulate endothelial cell migration and adherens junction assembly. The small GTPase Rac is critical for this receptor to activate the actin- and microtubule cytoskeleton dynamics. In contrast, the S1P2 receptor antagonizes this pathway by activating the small GTPase Rho/ and Rho-associated kinase (ROCK). Ultimately, this pathway induces the tumor suppressor phosphatase PTEN to inhibit cell migration and adherens junction assembly. Therefore, S1P receptors coordinately regulate angiogenesis and vascular permeability. In addition, mural cell recruitment to nascent vascular sprouts is also regulated by S1P1 signaling in vascular endothelial cells. Further understanding of S1P biology and signaling may avail novel therapeutic approaches in the control of vascular diseases.

724

**Protease-activated Receptors Play a Role in Intracellular $Ca^{2+}$ Dynamics of Arteriole Smooth Muscles of Brain**

Y. Satoh, T. Misaki, M. Matsuura, T. Saino, H. Akutsu-Yamauchi; Histology, Iwate Medical University, Morioka, Japan

[OBJECTIVE] Protease-activated receptors (PARs) mediate cellular responses to various proteases in diverse cell types, including smooth muscles and endothelium of blood vessels. PARs may play important roles in microcirculation in the tissue/organs. Here we examine whether stimulation of PARs induce responses of smooth muscles of arterioles. [MATERIALS AND METHODS] Arterioles (< 100 µm in diameter) were taken from brain and testis of rats. Isolated and cultured smooth muscles alter essential characteristics, therefore we used arteriole specimens which kept essential structural integrity. Intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) dynamics and nitric oxide (NO) production during PARs stimulations were evaluated by confocal microscopy (Nikon RCM/Ab), because $[Ca^{2+}]_i$ and NO are key factors in the maintenance of strain in blood vessels. [RESULTS] Thrombin and PAR1-agonist peptide (AP) induced oscillatory fluctuations of $[Ca^{2+}]_i$ of brain arteriole smooth muscles. The response was independent of extracellular $Ca^{2+}$, but depletion of intracellular $Ca^{2+}$ store suppressed the response. Small arterioles (< 50 µm in diameter) showed conspicuous oscillations, but larger those not. Any changes of endothelium were not verified. Trypsin and PAR2-AP induced faint oscillatory fluctuations and evident decrease of $[Ca^{2+}]_i$ level. It might be possible that activation of PAR2 induce NO production of endothelium, which in turn depressed $[Ca^{2+}]_i$ level of smooth muscle. However, NO production during PAR2 stimulation was not detected, and NO-synthase inhibitor did not suppressed PAR2 -AP induced $[Ca^{2+}]_i$ decrease. NO showed no decrease of $[Ca^{2+}]_i$ level. PAR3- and PAR4-AP had no effect. In contrast to brain arterioles, smooth muscles of testicular arterioles showed neither oscillatory fluctuations during PAR1 activation nor decrease of $[Ca^{2+}]_i$ level during PAR2 stimulation. [CONCLUSION] Proteases in various pathological conditions control microcirculation via PARs of arteriole smooth muscles in a NO-independent manner, although there may be organ-specificity.

725

**7-Ketocholesterol Predisposes Human Aorta Smooth Muscle Cell to Fas-mediated Death**

K. Kim, B. Rhim; Department of Pharmacology - School of Medicine, Pusan National University, Busan, Republic of Korea

Human vascular smooth muscle cells (HVSMCs) are resistant to Fas-mediated death under normal physiological conditions. However, HVSMC death by activation of the receptor pathway was reported in the atherosclerotic lesions. In this study, we investigated whether 7-ketocholesterol, one of the major cholesterol oxides in the lesions, altered resistance of HVSMC to Fas-mediated death pathway. Cross-linking of Fas receptor with agonistic anti-Fas antibody (CH11) in the presence of 7-ketocholesterol induced death in human aorta smooth muscle cells (HAoSMC) as detected by morphology, viability, and DNA fragmentation. The agonistic anti-Fas antibody, however, did not induce death in the presence of 7α-hydroxycholesterol or cholesterol. The HAoSMC death was significantly inhibited by an antagonistic Fas receptor (FasR) antibody and by expression of dominant negative Fas-associated death domain containing protein (DN-FADD) using adenoviruses. Activation of caspase-8 and -3 was observed in HAoSMC destined to death. HAoSMC death was significantly inhibited by pharmacological caspase inhibitor, z-VAD and z-DEVD, and baculovirus caspase inhibitor p35. Overexpression of bcl-xL also significantly inhibited HAoSMC death. The levels of Fas, FADD, caspase-8, caspase-10, caspase-3, caspase-9, c-FLIP, bax and XIAP were not changed in response to 7-ketocholesterol, whereas 7-ketocholesterol impaired mitochondrial transmembrane potential and ATP production. In dying HAoSMC, bax was translocated from the cytosol to mitochondria and cytochrome c was released from mitochondria into the cytosol. This is the first report demonstrating implication of the oxysterol in Fas-mediated death pathway. The present study proposes that 7-ketocholesterol would contribute to loss of HVSMC in the atherosclerotic lesions by altering resistance to receptor-mediated death pathway.

726

**Polarized Vascular Smooth Muscle Cell Mitoses in Response to Mechanical Load**

D. Dajnowiec,[1] J. T. K. Lam,[1] B. L. Langille[2]; [1]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada, [2]Cell and Molecular Biology, Toronto General Hospital, Toronto, ON, Canada

Arteries are constantly adapting to changes in mechanical forces imposed on them; therefore, they provide an excellent model for studying tissue responses to mechanical forces. We previously showed that vascular smooth muscle cells (VSMC) of the arterial wall mediate homeostatic regulation of longitudinal tension and we therefore hypothesize that mechanical tension induces VSMC to undergo polarized mitoses such that daughter cells are delivered in the direction of stress. To test this hypothesis, a segment of the left carotid artery of New Zealand White rabbits was surgically excised, and the cut ends of the vessel were reattached. BrdU, to label S-phase nuclei, was injected into the rabbits at surgery or 3 or 7 days later. The rabbits were euthanized at 1, 3, or 7 days after surgery and the LCA were perfusion-fixed at 100 mm Hg. Whole-mount preparations of the stretched vessel segments were prepared for en face immunohistochemistry and nuclear counter-staining, and mitotic orientations of newly divided nuclei doublets were analyzed using Laser Confocal Microscopy and image analysis software. Labeled nuclear doublets were oriented perpendicular to the arterial axis in control and sham-surgery vessels. In contrast, over 50% of the VSMC doublets oriented along the longitudinal axis in surgically-stretched LCA. Hence, the data indicate that normalization of tensile stretch in arteries involves the delivery of daughter VSMC in the axial direction by tension-induced polarized mitosis. Subsequent alterations in orientation and separation of daughter cell nuclei indicated that polarized mitosis was followed by cell migration that further modified position of cells within the vessel wall.

727

**Embryonic Lethality in Mice with Endothelial and Smooth Muscle Cell-Specific Deletion of the Bone Morphogenetic Protein Type IA Receptor (BMPR-IA)**

N. El-Bizri,[1] L. Wang,[1] E. Martinez,[1] C. P. Chang,[2] Y. Mishina,[3] M. Rabinovitch[1]; [1]Pediatrics, Stanford University, Stanford, CA, [2]Cardiovascular Medicine, Stanford University, Stanford, CA, [3]Molecular Developmental Biology Group, Stanford University, National Institute of Environmental Health Science, NC

Bmpr1a expression is reduced in lungs of patients with pulmonary arterial hypertension. (PAH). To determine the role of Bmpr1a in the pathogenesis of PAH, we selectively deleted Bmpr1a in vascular endothelial (EC) and smooth muscle cells. We bred mice expressing floxed Bmpr1a and ROSA26 reporter with Tie2-Cre and SM22-Cre mice, respectively. Both Tie2/Bmpr1a and SM22a/Bmpr1a homozygous knockout (-/- K/O) mice die around E11.5. While the cause of lethality of SM22/Bmpr1a -/- K/O is yet to be determined, vascular defects in yolk sac and placenta underlie the lethality of Tie2/Bmpr1a -/- K/O. At E11.5, the yolk sac was pale and devoid of blood. However, at E10.5, H&E stained cross sections revealed early signs of a degenerating vasculature manifest by detachment of the EC layer from the mesoderm. At E11.5, fusion of large and collapse of small vessels with total absence of blood cells was observed. Failure of penetration of embryonic tissue into the labyrinth of maternal placenta and fewer embryonic vessels were signs of defective placenta at E11.5. The E10.5 -/- K/O showed intact vasculature judged by whole-mount LacZ and PECAM staining. At E11.5 there was retardation in development and occasional enlargement of the pericardial cavity with a hemorrhagic effusion. Serial transverse sections at E9.5 and E10.5 excluded cardiac defects. Knocking down Bmpr1a by 72 % using RNAi in human pulmonary artery EC resulted in an attenuated serum-induced proliferative response ($P < 0.01$) judged by MTT assay but had no significant impact on migration. In conclusion, EC expression of Bmpr1a is necessary for normal yolk sac vascular remodeling and the impaired ability of EC to proliferate appears to be the consequence of loss of function of Bmpr1a in development and disease.

728

**A Novel Role for Fra-1 in Thrombin-induced Vascular Smooth Muscle Cell DNA Synthesis and Motility**

H. Cao, G. N. Rao; Department of Physiology, University of Tennessee Health Science Center, Memphis, TN

In order to understand the mechanisms by which thrombin induces vascular smooth muscle cell (VSMC) DNA synthesis and motility, here we have studied the role of PI3K-Akt-mTOR-S6K1 signaling. Thrombin stimulated the phosphorylation of Akt and S6K1 in VSMC in a sustained manner. LY294002, a specific inhibitor of PI3K, completely suppressed both Akt and S6K1 phosphorylation, whereas rapamycin, a specific inhibitor of mTOR, blocked only S6K1 phosphorylation induced by thrombin, suggesting that thrombin activates the PI3K-Akt-mTOR-S6K1 signaling in this order in VSMC. Blockade of PI3K-Akt-mTOR-S6K1 signaling by LY294002 and rapamycin prevented both thrombin-induced VSMC DNA synthesis and migration. Adenoviral-mediated expression of dominant negative Akt also inhibited thrombin-induced VSMC DNA synthesis and migration. Furthermore, thrombin induced the expression of Fra-1 in a sustained and PI3K/Akt-dependent and mTOR/S6K1-independent manner in VSMC. Suppression of Fra-1 by its siRNA attenuated both thrombin-induced VSMC DNA synthesis and migration. In addition, thrombin stimulated the tyrosine phosphorylation of epidermal growth factor receptor and inhibition of its kinase activity significantly blocked thrombin-induced Akt and S6K1 phosphorylation, Fra-1 expression and DNA synthesis in VSMC and their motility. Together, these observations suggest that thrombin induces both VSMC DNA synthesis and motility via EGFR-dependent stimulation of PI3K/Akt signaling targeting in parallel the Fra-1 expression and S6K1 activation.

729

**Examination of Endothelial Lumenization Processes *in vivo* Using Zebrafish *Danio rerio***

M. Kamei,[1] K. J. Bayless,[2] G. E. Davis,[2] B. M. Weinstein[1]; [1]LMG, NICHD/NIH, Bethesda, MD, [2]Department of Pathology and Laboratory Medicine, Texas A&M University System Health Science Center, College Station, TX

The process of vascular lumen formation has previously been studied in cultured endothelial cells *in vitro*. It has been shown that endothelial lumen formation occurs through accumulation of pinocytic vacuoles and their subsequent intra- and intercellular fusion . However, the relevance of data obtained through cell culture studies has not been tested in live animals before. Towards this end, we have prepared *Tg(fli1:cdc42wt-EGFP)* germline transgenic zebrafish in which a cdc42wt-EGFP fusion protein is expressed in endothelial cells *in vivo* under the control of the zebrafish *fli1* promoter. This *cdc42wt-EGFP* construct we used was previously shown to visualize vacuoles in endothelial cells in culture . We have shown that within *Tg(fli1:cdc42wt-EGFP)* zebrafish the cdc42wt-EGFP protein similarly localizes preferentially to the surface of cytoplasmic vacuolar structures. By using this transgenic line, we have captured dynamic images *in vivo* of lumenization of the developing blood vessel. The images show that the cytoplasmic vacuolar structures coalesce into the mature vascular endothelial lumen *in vivo.* These images suggest that lumenization *in vivo* also occurs in a very similar manner to that of the vacuole formation and fusion process shown *in vitro*. References

730

**Role of Hepatocyte Growth Factor (HGF) in Expression of Proteases and Cell Migration during Retinal Neovascularization**

E. Colombo; Cell Biology and Physiology, University of New Mexico, School of Medicine, Albuquerque, NM

**Purpose:** Hepatocyte growth factor (HGF), and its receptor, c-met, are known to promote cell migration. They have been implicated in the progression of angiogenesis in many cancer models and are upregulated in diabetes. However, HGF mediation of protease expression and endothelial cell migration and invasion in the diabetic retina has not been explored. We determined if HGF affects cell migration and protease levels in retinal microvascular endothelial cells and in a mouse model of retinal neovascularization. **Methods:** Bovine retinal microvascular endothelial cells (BRMVEC) were stimulated with HGF (10ng/ml). At 24 hours, conditioned media and cell extracts were collected and analyzed by zymography or by reverse-transcription PCR. Migration and invasion assays were performed with BRMVEC's grown on migration chambers with or without Matrigel. HGF or VEGF was used in the bottom chamber as a chemoattractant. After 24 hours, the amount of migration into the lower chamber was analyzed. Retinal neovascularization was induced in newborn mice by exposure to 75% oxygen followed by room air. Eyes were collected at day 17 following removal from high oxygen, fixed, and paraffin embedded. Immunohistochemistry was performed on sections to localize retinal c-met expression. **Results:** After 24 hours of HGF stimulation, there was a significant increase in endothelial cell expression of urokinase. PCR analysis of uPA and uPAR mRNA showed a similar expression pattern. Migration and invasion assays demonstrated that HGF

significantly increased BRMVEC cell migration and invasion compared to VEGF samples. Immunohistochemical staining showed localized c-Met expression in the retina, which was increased in experimental animals compared to control. **Conclusion:** HGF, acting through its receptor c-met, may play an important role in the initial stages of retinal angiogenesis by stimulating a migratory phenotype and endothelial cell protease production.

731

## Low-dose Irradiation Promotes Tissue Revascularization Through VEGF Release from Mast Cells and Mmp-9 Mediated Progenitor Cell Mobilization

B. Heissig,[1] H. Akiyama,[1] S. Rafii,[2] Z. Werb,[3] K. Hattori[1]; [1]Stem Cell Regulation, Center of Experimental Medicine, University of Tokyo, Tokyo, Japan, [2]Dep. of Hematology-, Cornell University, Tokyo, Japan, [3]Dep. of Anatomy, University of California, San Francisco, CA

Mast cells accumulate in tissues undergoing angiogenesis during tumor growth, wound healing, and tissue repair. Mast cells can secrete angiogenic factors such as vascular endothelial growth factor (VEGF). Ionizing irradiation has angiogenic potential in malignant and non-malignant diseases. We observed that low-dose irradiation fosters mast cell-dependent vascular regeneration in a limb ischemia model. Irradiation promoted VEGF production by mast cells in a matrix metalloproteinase-9 (MMP-9) dependent manner. Irradiation, through MMP-9 upregulated by VEGF in stromal and endothelial cells, induced the release of Kit-ligand (KitL). Irradiation-induced VEGF promoted migration of mast cells from the bone marrow to the ischemic site. Irradiation-mediated release of KitL and VEGF was impaired in MMP-9 deficient mice resulting in a reduced number of tissue mast cells and delayed vessel formation in the ischemic limb. Mast cell deficiency steel mutant ($Sl/Sl^d$ mice) or blockade of the VEGF pathway abrogated irradiation-induced vasculogenesis. Irradiation did not induce progenitor mobilization in $Sl/Sl^d$ mice. We conclude that increased recruitment and activation of mast cells following irradiation alters the ischemic microenvironment and promotes vascular regeneration in an ischemia model. These data show a novel mechanism of neovascularization and suggest that low-dose irradiation may be used for therapeutic angiogenesis to augment vasculogenesis in ischemic tissues.

732

## Signal Transduction Pathways Stimulated by Multiple Angiogenic Factors Result in Activation of SREBPs-HMGCoA-RhoA

M. Yao, R. Zhou, M. Petreaca, M. Martins-Green; Cell Biology & Neuroscience, UCR, Riverside, CA

Angiogenesis is essential in many physiological and pathological processes. Therefore, to better understand the angiogenic process and to more efficiently manipulate it, it is important to identify common signaling pathways stimulated by different angiogenic factors. Sterol regulatory element-binding proteins (SREBPs) are transcription factors regulating the metabolism of cholesterol and fatty acids, which are critical in membrane biology and signal transduction. In previous studies, we have shown that VEGF activation of SREBPs plays a key role in angiogenesis. In this study, using human microvascular endothelial cells (hMVEC) and the Chorioallantoic membrane (CAM) assay, we further show that angiogenic factors belonging to different families, such as bFGF, thrombin, and IL-8, also stimulate SREBP activation despite their activation of disparate classes of receptors (bFGF through tyrosine kinase receptors, IL-8 and thrombin through G-protein-coupled receptors). In contrast non-angiogenic factors, such as TGFβ1, do not. This activation of SREBPs is critical in permeability of the endothelium and in endothelial cell migration, as well as IL-8-induced angiogenesis *in vivo*. Furthermore, we explored the underlying mechanism and found that the effects of SREBPs on angiogenesis stem, at least in part, from their regulation of RhoA geranylgeranylation by geranylgeranyl pyrophosphate (GGPP), a product downstream of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) during cholesterol biosynthesis. Taken together, our data suggest that the HMGCoA-RhoA activation is one of the signal transduction events occurring downstream of SREBP in the angiogenesis induced by two very different angiogenic factors, IL-8 and VEGF. Given that diverse angiogenic factors use different types of cell-surface receptors, identification of this common signal transduction pathway as a target could provide the opportunity for novel approaches to the prevention and treatment of diseases involving abnormal angiogenesis.

733

## Hepatic Stellate Cells in Arctic Top Predators

H. Senoo,[1] K. Wake,[2] H. L. Wold,[3] N. Kuwata-Higashi,[1] T. Irie,[1] K. Yoshikawa,[1] J. Ø. Moskaug,[3] N. Kojima,[1] M. Miura,[1] K. Imai,[1] M. Sato,[1] N. Roos,[4] T. Berg,[4] K. R. Norum,[3] R. Blomhoff[3]; [1]Department of Cell Biology and Histology, Akita University School of Medicine, Akita, Japan, [2]Liver Research Unit, Minophagen Pharmaceutical Co. Ltd., Tokyo, Japan, [3]Institute for Nutrition Research, University of Oslo, Faculty of Medicine, Oslo, Norway, [4]Department of Biology, University of Oslo, Oslo, Norway

After getting permission to hunt the animals from the district governor of Svalbard, 11 arctic foxes, 14 bearded seals, 22 glaucous gulls, 5 fulmars, 4 Brünnich's guillemots, 6 ringed seals, 5 hooded seals, 6 puffins, 5 Svalbard ptarmigans and 7 Svalbard reindeers were caught in the period from August 1996 to September 2001. Three polar bears were shot in self-defense at Svalbard February and August 1998 in Ny Ålesund and Hornsund. Four polar bears were caught in August 2004 in Greenland. We also obtained 13 brown bears from Jämtland, Gävleborg and Dalarna, 4 red foxes from Västergötaland, and 8 grey gulls from Skåne, Sweden. Fresh organs, namely, the liver, kidney, spleen, lung, and jejunum were examined by morphological methods such as gold chloride method, fluorescence microscopy for detection of vitamin A autofluorescence, and transmission electron microscopy, and high-performance liquid chromatography. The arctic animals stored vitamin A in hepatic stellate cells. Only a small amount of vitamin A existed within other organs such as kidney, spleen, lung, and jejunum. Top predators among arctic animals stored 6-23 micro mole retinyl ester per g liver which 20-100 times the levels normally found in other animals including humans. These results indicate that the hepatic stellate cells in these animals have high ability for uptake and enough capacity for storage of vitamin A. The liver lobules of the arctic animals showed a zonal gradient in the storage of vitamin A. The gradient was expressed as a symmetric crescendo-decrescendo profile starting at the peripheral zone in the lobule. The density of hepatic stellate cells was essentially the same among the zone. These results indicate that the heterogeneity of vitamin A-storage capacity in hepatic stellate cells in arctic animals is genetically determined.

## Wiring the Nervous System (734-736)

734

### Mechanism of Presynaptic Assembly

Y. Jin; Molecular, Cellular & Developmental Biology, Howard Hughes Medical Institute, University of California, Santa Cruz, CA

Synapses are asymmetric cellular junctions composed of presynaptic and postsynaptic specializations. At the presynaptic terminal synaptic vesicles accumulate in an ordered fashion and docked around the electron-dense active zone. To examine the molecular mechanism controlling the formation of the cytoarchitecture of presynaptic terminals, we undertake a genetic approach in the nematode *C. elegans*. Analyses of the mutants that exhibit altered synapse structures have led the identification of conserved molecules that function to define distinct spatial domains at presynaptic terminals. Further genetic interaction studies reveal multiple signal transduction pathways involving ubiquitin-mediated protein degradation and MAP kinases play crucial roles in controlling synaptic integrity.

735

### Activity-dependent Development of Cortical Connections

A. Ghosh; University of California, San Diego, La Jolla, CA

One of the most remarkable features of the nervous system is that its development is controlled by sensory experience. The effect of experience on brain development is mediated by synaptic activity, which leads to calcium entry into the postsynaptic neuron. The mechanisms by which calcium signaling leads to lasting changes in neural circuits has been extensively investigated over the past two decades, and involves both transient and sustained biochemical changes. Whereas rapid changes in synaptic circuits are mediated by post-translational modification of proteins located near the site of calcium entry, long-term changes require new gene expression. To identify transcriptional mediators of activity-dependent development, we developed a strategy called Transactivator Trap to clone calcium-activated transcription factors in neurons. Two of these factors, CREST and NeuroD2, regulate distinct aspects of activity-dependent development. We will present molecular and genetic evidence that CREST, a CBP-binding protein, plays a key role in mediating activity-dependent development of cortical dendrites, and that NeuroD2 plays a critical role in synapse maturation and the patterning of thalamo-cortical connections. These findings advance our understanding of the transcriptional mechanisms by which sensory experience regulates brain development.

736

### Effects of Experience on Visual System Development

H. Cline; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

We examined the effect of visual experience on the development of the retinotectal system in Xenopus tadpoles. Dendritic and axonal arbors of developing neurons grow by the stabilization of a small fraction of highly dynamic branches. To test the effect of synapse maturation on axon arbor development, we co-expressed cytosolic fluorescent protein and fluorescent protein tagged synaptophysin (SYP) in retinal cells to reveal presynaptic sites within labeled retinotectal axons. Two-photon time lapse observations demonstrated that synaptic contacts stabilize retracting branches. Patterned visual activity stabilized retinal axon arbor structure by reducing the extension of new branches and by increasing retractions of branches to sites stabilized by strong synapses. To test whether glutamatergic synaptic maturation affects the development of dendritic arbors, we interfered with AMPA receptor trafficking into synapses by expressing the intracellular C-terminal domains of AMPA receptor subunits GluR1 or GluR2 in individual optic tectal neurons. This reduced AMPA mEPSC amplitude by 50% and dramatically altered dendritic arbor growth over 5 days, resulting in less complex arbors. Time-lapse images show that expression of GluR C-terminal domains decreased the stability of dendritic branches, suggesting that glutamatergic synapse maturation stabilizes dendritic branches. In control GFP-expressing neurons, visual stimulation increases dendritic arbor growth by increasing branch stabilization. Expression of GluR C-terminal domains completely blocked the experience-dependent increase in dendritic arbor growth rates. These data demonstrate that visual stimulation affects retinotectal development by differentially influencing afferents and dendrites: experience consolidates axon arbor structure by restricting branch extensions and retractions, whereas experience promotes dendritic arbor growth by stabilizing newly added dendritic branches.

## The Nature of Life: An Orientation Course that Introduces Freshmen to the Disciplines of Biology, Builds Community, and Teaches Strategies for Success in College (737)

737

### The Nature of Life: An Orientation Course That Introduces Freshmen to the Disciplines of Biology, Builds Community, and Teaches Strategies for Success in College

R. Wright, J. S. Anderson, D. Biesboer, S. Cotner, S. Gilbert; College of Biological Sciences, University of Minnesota, Minneapolis, MN

The University of Minnesota aims to increase the retention and graduation rates of entering freshmen. To help accomplish this goal, the College of Biological Sciences has developed a one-credit orientation course called “The Nature of Life” that is required for all of its incoming freshmen, a class of ~350 students. The Nature of Life program introduces students to the breadth of biological disciplines, acquaints them with college and university resources, and helps them become integrated into a community of learners. The program begins with a four-day experience at the Itasca Biological Station and Laboratories located at the headwaters of the Mississippi River. During this time, students participate in three different interactive learning modules that focus on topics ranging from bioethics and neurobiology to aquatic ecosystems and spatial modeling. Additional programming includes plenary lectures and discussions about career planning, time management, research involvement, campus resources, and University of Minnesota traditions. A college-level exam is given, graded, and returned to students on the last evening of the program, followed by a discussion of strategies for more effective learning. To receive credit for the course, students must also prepare three reflective essays, attend events on campus, and participate in discussions of articles from a popular science magazine. First semester freshmen who participated in the Nature of Life program have higher overall GPAs and fewer failing grades than those of previous years. In addition, one year retention in the college increased from 66% to 81%. To our knowledge, this program is unique among research universities in the United States and provides a

model for supporting a successful transition of students from high school into the academic challenges of the university.

## Adapting to Stress: Spotlight on Organelles (738-740)

738

### A Novel Class of Membrane Proteins Shapes the Tubular Endoplasmic Reticulum

G. K. Voeltz,[1] W. A. Prinz,[2] J. M. Rist,[3] T. A. Rapoport[1]; [1]Department of Cell Biology, HHMI/Harvard Medical School, Boston, MA, [2]Laboratory of Cell Biochemisry and Biology, NIDDK/National Institutes of Health, Bethesda, MD, [3]Department of Cell Biology, Harvard Medical School, Boston, MA

How is the characteristic shape of a membrane-bound organelle achieved? We have used an in vitro system to address the mechanism by which the tubular network of the endoplasmic reticulum (ER) is generated and maintained. Based on the inhibitory effect of sulfhydryl reagents and antibodies, we demonstrate that network formation in vitro requires the integral membrane protein Rtn4a/NogoA, a member of the ubiquitous reticulon family. Both in yeast and mammalian cells, the reticulons are largely restricted to the tubular ER and excluded from the continuous sheets of the nuclear envelope and peripheral ER. Upon overexpression, the reticulons form tubular membrane structures. The reticulons interact with DP-1/Yop1p, a conserved integral membrane protein that also localizes to the tubular ER. The simultaneous absence of the reticulons and Yop1p in S. cerevisiae results in disrupted tubular ER. We propose that these proteins stabilize ER tubules by utilizing a common hairpin structure to partition into and stabilize highly curved membranes.

739

### Controlling Endoplasmic Reticulum Client Protein Load

D. Ron; Skirball Institute, New York University School of Medicine, New York, NY

In eukaryotic cells the vast majority of secreted and membrane bound proteins are synthesized in association with endoplasmic reticulum (ER) membranes. Newly-synthesized proteins, or segments thereof, are translocated into the lumen of the ER where they are acted on by an elaborate post-translational protein-handling machinery unique to that organelle. That machinery affects various post-translational modifications in ER client proteins, promotes the folding of individual client polypeptides and assembles multimeric complexes from individual subunits. It is also identifies terminally-misfolded proteins and promotes their destruction. The equilibrium between the ER's protein handling machinery and the load of client proteins is maintained by a dedicated set of signal transduction pathways collectively referred to as the Unfolded Protein Response (UPR). The UPR culminates in two major adaptations to a shift in the equilibrium favoring unfolded client proteins (a shift referred to heuristically as ER stress): (i) Activation of a transcriptional program that enhances the organelle's capacity to deal with client proteins. (ii) Attenuation of the influx of client proteins into the lumen of the endoplasmic reticulum. In this presentation we will focus on the second adaptation. We will review the molecular mechanisms underlying cell's ability to control the flux of client proteins into the ER and will discuss various examples of the consequences of failures in this "primary prevention" strategy against ER stress.

740

### An Unexpected Role of Bcl-2 Family Members in Mitochondrial Morphogenesis

R. J. Youle; Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD

Bax, a member of the Bcl-2 family, is required for the normal program of cell death during mammalian development. Early during apoptosis, as an essential step in cell death promotion, Bax translocates from the cytosol to mitochondria almost simultaneously with a dramatic fragmentation of the normal mitochondrial network. Subsequent to translocation to the outer mitochondrial membrane, Bax coalesces into huge complexes on the surface of mitochondria at sites that subsequently constrict and become points of mitochondrial fission. The mitochondrial fission and fusion machinery is comprised of several proteins including dynamin related protein 1 (Drp1) and mitofusin 2 (Mfn2) both of which colocalize with Bax in foci at prospective mitochondria fission sites. Inhibition of mitochondrial fission inhibits apoptosis further suggesting that Bax may participate in a specialized mitochondrial fission process during apoptosis. An endophilin isoform that binds to Bax during apoptosis is normally involved in regulating mitochondrial morphology in healthy cells. Using RNA interference to deplete cells of endophilin B1 causes some dissociation of the outer mitochondrial membrane compartment from that of the matrix and the formation of thin, elongated tubules of outer mitochondrial membrane. Knock down of Drp1 in addition to knock down of endophilin B1 leads to a mitochondrial phenotype identical to that of the Drp1 single knock down, a result consistent with Drp1 acting upstream of endophilin B1 in the same pathway of mitochondrial division. Thus, mitochondrial fission requires two proteins, Drp1 and endophilin B1, homologous to two members of the synaptic vesicle recycling system pointing to an unanticipated relationship in the mechanism of these two processes. Interestingly, knock down of endophilin B1 also confers cellular resistance to a number of apoptosis pathways suggesting that it may participate with Bax in outer mitochondrial membrane remodeling during cell death.

## Cell Migration/Motility (741-746)

741

### Vesicular Trafficking And Signal Relay During Chemotaxis

P. W. Kriebel, V. A. Barr, V. A. Mihaylov, C. A. Parent; Laboratory of Cellular and Molecular Biology, National Cancer Institute/NIH, Bethesda, MD

The social amoebae *Dictyostelium*, human leukocytes, as well as many other types of cells possess the ability to polarize and move in a directed fashion in response to chemoattractants. In *Dictyostelium* the most potent chemoattractant is cAMP, which like chemokines, transduces its effects by binding to G protein-coupled receptors. The binding of cAMP to the cAMP receptor cAR1 activates a variety of effectors, including the activation of the adenylyl cyclase ACA, which converts ATP to cAMP. The majority of cAMP is then secreted to relay the chemoattractant signal to neighboring cells. Using the GFP technology, we have shown that ACA is highly enriched at the back of chemotaxing cells. We propose that this provides a compartment from which cAMP is secreted allowing cells to orient themselves and form chains of cells or streams. In addition to its localization at the plasma membrane, we find that ACA exists in an internal pool of vesicles that show rapid dynamic movement. These vesicles do

not co-localize with ER or Golgi markers. Similarly, Alexa594-dextran or Alexa594-BSA, which label the lysosomal pathway, do not co-localize with ACA. We investigated whether these vesicles are involved in the trafficking of ACA using photo-bleaching studies. We bleached a small box encompassing the plasma membrane and monitored the fluorescence recovery. While the cAR1-GFP recovery occurs first in the edges of the bleached box, we find that the ACA-YFP fluorescence recovery occurs uniformly on the plasma membrane and, most remarkably, that the extent of recovery increases and is targeted to the back of the cells as they differentiate and polarize. These results suggest that ACA is replenished via vesicles fusion to the plasma membrane. We propose that membrane trafficking is part of a regulatory mechanism that controls the activity of ACA.

742

**Posttranslational N-terminal Arginylation Regulates Cell Motility and Actin Cytoskeleton**

M. Karakozova,[1] M. Kozak,[1] R. Rai,[1] C. C. L. Wong,[2] A. O. Bailey,[2] J. R. Yates,[2] A. Kashina[1]; [1]Department of Animal Biology, University of Pennsylvania, Philadelphia, PA, [2]The Scripps Research Institute, LaJolla, CA

N-terminal arginylation is a poorly understood posttranslational modification that is critical for embryogenesis, cardiovascular development, and angiogenesis in mammals. To understand the physiological role of protein arginylation, we developed a screen to identify proteins arginylated in vivo. We found that actin and several other major components involved in cytoskeleton structure and regulation are directly regulated by arginylation. Several properties of actin, including its ability to bind actin-associated proteins, are affected by arginylation and altered in arginylation-deficient actin preparations. Analysis of the morphology and behavior of cultured embryonic fibroblasts derived from the ATE1 knockout mice indicates that arginylation regulates cell motility and adhesion by affecting cytoskeleton, focal adhesions, and extracellular matrix, and that the absence of ATE1 results in the inability of cultured cells to form a lamella and impairment of their directional migration across the substrate. These changes appear to be directly linked to the corresponding arginylation targets and suggest a new mechanism for the regulation of cytoskeleton and cell motility that can explain the observed effects of ATE1 knockout in vivo.

743

**IQGAP1-Stimulated Actin Assembly Linked to Growth Factor Receptor Activation**

L. B. Benseñor, N. Wang, H. M. Kan, S. C. Mateer, L. E. Morris, D. A. Schafer, G. S. Bloom; Department of Biology, University of Virginia, Charlottesville, VA

IQGAP1 is an actin-binding protein that concentrates at sites of branched actin filament assembly at leading edges of migratory cells. In endothelial cells, IQGAP1 binds the cytoplasmic tail of VEGFR2 after receptor activation by VEGF, and siRNA-induced reduction of IQGAP1 inhibits VEGF-stimulated cell motility (Yamaoka-Tojo, et al. 2004. Circ. Res. 95: 276-283). We now present evidence that this motility requirement for IQGAP1 reflects the ability of IQGAP1 to stimulate branched actin filament assembly, and that in non-endothelial cells, which lack VEGFR2, IQGAP1 works through at least one other receptor, FGFR1. In vitro assays with purified proteins demonstrate that IQGAP1 stimulates branched actin filament assembly comparably to activated Cdc42 in the presence of N-WASP and Arp2/3 complex, and acts additively with Cdc42 to stimulate polymerization kinetics further and reduce the lag that precedes maximal F-actin assembly rates. Actin assembly is not stimulated by IQGAP1 fragments lacking the actin-binding region or more C-terminal domains, or by full length IQGAP1 that is prevented from binding actin by calmodulin. Studies of CV-1 and MDBK cells stimulated with serum or bFGF establish in vivo relevance for these results. Serum stimulation leads to rapid recruitment of IQGAP1, N-WASP, Arp3 and FGFR1 to lamellipodia, as judged by immunofluorescence, and to time-dependent increases in levels of N-WASP, Arp3 and FGFR1 that co-IP with IQGAP1. Stimulation with bFGF similarly causes recruitment of FGFR1 and IQGAP1 to lamellipodia. These results establish IQGAP1 as a major, but previously unrecognized regulator of actin assembly and cell motility mediated by Arp2/3 complex and N-WASP, and imply that this property of IQGAP1 is coupled to growth factor receptor signaling.

744

**Intracellular Fluid Dynamics in Motile Fish Keratocytes**

K. Keren,[1] B. Hebert,[2] T. F. Harper,[3] P. W. Wiseman,[2,4] A. Mogilner,[5] J. A. Theriot[1]; [1]Dept. of Biochemistry, Stanford University, Stanford, CA, [2]Dept. of Physics, McGill University, Montreal, PQ, Canada, [3]Quantum Dot Corporation, Hayward, CA, [4]Dept. of Chemistry, McGill University, Montreal, PQ, Canada, [5]Dept. of Mathematics and Center for Genetics and Development, University of California, Davis, CA

Molecular mechanisms and biophysical processes are intimately intertwined to generate the robust large-scale self-organization manifested by a moving cell. While much research has been devoted to the molecular mechanisms underlying cell motility, less is known about its biophysical aspects such as the intracellular fluid dynamics. We are measuring the flow and effective diffusion in the fluid phase of moving cells, which are important biophysical parameters, as well as being a significant determinant for the intracellular transport of molecular components. In order to study the fluid dynamics inside living cells we introduce polyethylene glycol-coated quantum dots (QDs) as inert tracers into fish epidermal keratocytes. We follow the motion of the QDs by live-cell fluorescence microscopy, and extract their behavior by both spatio temporal image correlation spectroscopy and single particle tracking. Moving cells exhibit a directed fluid flow in the lamellipodium away from the cell's leading edge and back towards the cell body at a speed ~30% of the cell movement speed. To further understand the relationship between intracellular fluid flow and cell motility we use various perturbations which are known to alter the fluid flow through the cell membrane (e.g. Hg, 5-(N-ethyl-N-isopropyl) amiloride and osmolarity shifts) or the properties of the actin meshwork (e.g. blebbistatin) and study their effect. We find that fluid flow does not have a significant contribution to the transport of essential components of the actin machinery to the leading edge in keratocytes, and that this transport is largely maintained by random diffusive-like motion. The measured fluid flow seems to concur with theoretical predictions based on a mathematical model of keratocyte motility.

745

**A Novel Function for the Polarity Regulator Scribble in T Lymphocyte and Epithelial Cell Migration**

L. E. Dow, M. J. Ludford-Menting, J. Oliaro, F. Sacirbegovic, J. Kauffman, R. M. Iazzolino, S. M. Russell, P. O. Humbert; Research Division, Peter MacCallum Cancer Centre, Melbourne, Australia

Analyses of *Drosophila melanogaster*, zebrafish and mouse mutants have highlighted an important role for the polarity regulator Scribble in the

organization of epithelial tissue during embryonic development. In particular, mutation of Scribble in *Drosophila* epithelium gives rise to loss of apical-basal cell polarity and tissue overgrowth. As such, Scribble has classically been defined as a neoplastic tumour suppressor. We have utilized a combination of mammalian and *Drosophila* systems to identify and characterize the cellular functions of Scribble in development and cancer. Here we show a novel conserved function for Scribble in the regulation of T lymphocyte and epithelial cell migration. We show that in T lymphocytes, Scribble and associated polarity proteins polarize to the uropod of migrating T cells and disruption of the Scribble complex prevents uropod formation and inhibits migration. In mammary epithelial cells, Scribble is recruited to the leading edge of migrating cells and loss of Scribble results in impaired directed migration. Scribble-depleted epithelial cells fail to re-orient their MTOC/Golgi in response to directional migration cues and show disruption of the microtubule network and decreased Rac-1 containing actin rich protrusions at their leading edge. Importantly, Scribble physically interacts with regulators of RhoGTPases in both T lymphocytes and mammary epithelial cells. We propose a model by which Scribble coordinates directional migration through the regulation of the polarised distribution of the machinery required for cell movement.

746

**Plasticity of tumor invasion: Molecular mechanisms of mesenchymal-amoeboid transition after loss of integrin function**

P. Friedl; Cell Migration Laboratory, Department of Dermatology, University of Würzburg, Würzburg, Germany

Tumor cell migration through connective tissue requires adhesive cell matrix interactions provided by surface integrins and other adhesion systems. We have investigated, whether tumor cell migration in 3D ECM environments is fully abrogated by blocking integrin functions and whether migratory rescue may occur. Within 3D collagen matrices, invasive MV3 melanoma cells preferentially utilize α2β1 integrins for elongation, adhesion to collagen fibers, fiber bundling, force generation, and migration. Using these cells, β1 integrin function was reduced by i) flow cytometric sorting for subsets expressing low integrin levels; ii) blocking anti β1 mAb 4B4 at different concentration; iii) rhodocetin, a selective α2β1 disintegrin. Lowering β1 integrin function resulted in a varying yet incomplete reduction of migration rates accompanied by the conversion from fibroblastlike spindle-shaped morphology to more spherical amoeboid morphodynamics, and concomitantly abolished capacity to contract and remodel 3D collagen lattices. Residual amoeboid migration efficiencies amounted to undiminished 0,3- 0,4 µm/min in faster and 0,03-0,1 µm/min in slower cell subsets, sustained by propulsion and squeezing along matrix gaps provided by a strictly cortical actin cytoskeleton but lacking integrin and actin focalization at cell-matrix interactions. These findings were confirmed for fibroblast-like cells derived from β1-/- ES cells (GD25) and β1-/- murine fibroblasts, both of which exhibited slow (GD25) or fast amoeboid movement (β1-/- fibroblasts) after β1 integrin deletion. After simultaneous inhibition of ß1, αvβ3, and GAG-mediated cell-ECM interactions the residual migration was abolished in >60% of the cells and 30% exhibitng minimal residual movement below 0.02 µm/min. In conclusion, mesenchymal-amoeboid transition after abrogation of integrin function is a rescue mechanism supporting residual cell migration by low- or non- adhesive mechanisms through the surface glycocalyx.

## Chromatin Dynamics (747-752)

747

**Condensin I Stabilizes Chromosomes Mechanically Through a Dynamic Interaction in Live Cells**

D. Gerlich,[1] T. Hirota,[2] B. Koch,[2] J. M. Peters,[2] J. Ellenberg[3]; [1]Institute of Biochemistry, ETH Zuerich, Zuerich, Switzerland, [2]Research Institute of Molecular Pathology, Vienna, Austria, [3]Gene Expression and Cell Biology/Biophysics Programmes, EMBL Heidelberg, Heidelberg, Germany

Restructuring chromatin into morphologically distinct chromosomes is essential for cell division, but the molecular mechanisms underlying this process are poorly understood. Here, we investigated the function of the proposed key factors condensins, functionally tagged with GFP, in live cells. Using fluorescence photobleaching and quantitative time-lapse imaging, we found that condensin II bound stably to chromosomes throughout mitosis. By contrast, the canonical condensin I was not present on chromosomes until the completion of prophase condensation. After nuclear envelope breakdown, condensin I rapidly associated with chromosomes through a dynamic interaction, reaching steady state levels before congression. In condensin I depleted cells, compaction was normal, but chromosomes were mechanically labile and unable to withstand spindle forces during alignment. We propose that chromosome condensation is a stepwise process of compacting highly plastic chromatin in prophase, in part mediated by condensin II, followed by locking the condensed chromatin in a rigid state through rapid condensin I binding after nuclear envelope breakdown, which prepares chromosomes for subsequent spindle attachment.

748

**A Family of Site-Specific Binding Proteins Controls Meiotic Chromosome Pairing and Segregation in *C elegans***

C. M. Phillips,[1] A. Sato,[2] C. Wong,[1] A. F. Dernburg[1,3]; [1]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [2]Graduate School of Biostudies, University of Kyoto, Kyoto, Japan, [3]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

Physical interactions between homologous chromosomes are essential for genetic recombination and accurate segregation during meiosis. To understand the mechanisms underlying meiotic chromosome dynamics, we have focused on the roles of specific chromosome sites known as Pairing Centers, which we have shown to mediate homolog pairing and synapsis in *C. elegans*. Through genetic interactions with Pairing Center mutations, we identified a gene, *him-8*, which is specifically required for *X* chromosome segregation. We cloned *him-8* and learned that it encodes a C2H2 zinc finger protein that associates specifically with the Pairing Center of the X chromosome and also with the nuclear envelope. Identification of *him-8* also revealed that it is just one member of a multi-gene family transcribed from the same operon. Cytological analysis of the localization of the other family members has revealed that each protein is expressed during meiosis, localizes to the nuclear envelope, and binds to the Pairing Center(s) of either one or two specific autosomes. We have also observed colocalization of these proteins with patches of SUN-1 and ZYG-12, two transmembrane proteins that are thought to bridge the inner and outer nuclear envelope and to mediate centrosome attachment. These observations suggest a role for cytoskeletal components in homologous chromosome pairing and synapsis.

749

**Purification of Native Silent Chromatin in Budding Yeast**

A. D. Rudner, S. Gerber, S. Gygi, D. Moazed; Cell Biology, Harvard Medical School, Boston, MA

Silent chromatin, or heterochromatin, is found at telomeres, the *HM* loci and rDNA repeats in budding yeast. Like heterochromatin in other organisms, silent chromatin is a repressive chromatin structure that is late replicating, refractory to transcription and recombination, and is epigenetically inherited. Silent chromatin in budding yeast is thought to assemble from a complex of Sir2, Sir3 and Sir4 (the SIR complex) spreading along hypoacetylated regions of chromatin. An analogous structure exists in fission yeast and larger organisms where the Swi6 (an HP1 homologue) protein spreads along regions of chromatin that are methylated at lysine 9 of histone H3. Genetic screens have identified other proteins that are involved in initiating the assembly of silent chromatin, but no method has allowed a systematic identification of the complete set of proteins physically present in this repressive chromatin domain. In order to determine the precise structure and protein composition of silent chromatin we have developed a method of biochemically purifying fragments of native silent chromatin. As expected, these fragments contain the SIR complex, stoichiometric quantities of nucleosomes, as well as proteins that initiate silent chromatin assembly. In addition we find a large group of known chromatin proteins as well as others with unknown roles in chromosome metabolism. One co-purifying protein is the protein Asf2 that we have determined is a core component of silent chromatin and binds tightly to the SIR complex. We suspect that Asf2 may play a role in disassembling silent chromatin because overexpression of *ASF2* disrupts both silencing and the formation of the SIR complex in vivo. We are currently studying the mechanism by which Asf2 disassembles silent chromatin and the physiological processes that require such disassembly.

750

**ATP-dependent Nucleosome Remodelling Factors: Motors for Epigenetic Inheritance**

P. D. Varga-Weisz; Babraham Institute, Cambridge, United Kingdom

ATP-dependent nucleosome remodelling factors are key mediators of chromatin dynamics involved in transcription regulation, DNA- replication and repair. Our work indicates a role for these factors in determining chromatin structure right at the DNA replication fork. The nucleosome mobilizing ATPase Imitation Switch (ISWI) is an important nucleosome remodelling factor in mammalian cells. It forms several functionally and biochemically distinct remodelling factors by interacting with other proteins. One of these is the Williams-Syndrome Transcription Factor, WSTF. We observed that the ATP-dependent nucleosome remodelling complex WICH (WSTF-ISWI Chromatin remodelling factor) is targeted to replication sites via replication molecule PCNA and determines the structure of newly replicated chromatin. Depletion of WSTF results in formation of aberrant heterochromatin like structures and impairs transcription. We propose a model that postulates that the action of remodelling factors, such as WICH, is required right at the replication fork to facilitate the rebinding of factors that determine chromatin structure and transcriptional status. Thus, such factors facilitate the maintenance of chromatin structures from the parental DNA to the daughter DNA strands. Currently we are testing if other ATP-dependent nucleosome remodelling factors may function in a similar mode. In fission yeast, we do not find orthologues of ISWI, but a member of another class of ATP-dependent nucleosome remodelling factors may target the replication site there to determine chromatin structure. References: Poot RA, Bozhenok L, van den Berg DL, Steffensen S, Ferreira F, Grimaldi M, Gilbert N, Ferreira J, Varga-Weisz PD. The Williams syndrome transcription factor interacts with PCNA to target chromatin remodelling by ISWI to replication foci. Nat Cell Biol. 2004 Dec;6(12):1236-44. Poot RA, Bozhenok L, Nicola Hawkes Varga-Weisz PD. Chromatin Remodeling by WSTF-ISWI at the Replication Site: opening a Window of Opportunity for Epigenetic Inheritance? Cell Cycle, 4 (4) 543-6.

751

**Mechanisms of Activation and Silencing of the Immunoglobulin Kappa Locus through DNA Looping and Nuclear Repositioning to Heterochromatin**

Z. Liu, P. Widlak, Y. Zou, M. Oh, M. Chang, J. W. Shay, W. T. Garrard; Molecular Biology, UTSouthwestern Medical Center, Dallas, TX

We have investigated the higher-order chromatin structure of the mouse immunoglobulin (Ig) kappa locus as a function of activation and silencing during B cell development using the techniques of chromosome conformation capture (3C) and 3D 3-color FISH. We find that in the fully transcriptionally active state that each of the three enhancers form complexes with themselves and rearranged gene promoters, while the resulting intervening DNA is looped out. These results fit the looping model for the mechanism of enhancer action. Using chromatin immunoprecipitation in combination with 3C (e.g., ChIP-3C), we demonstrate that the transcription factor E47 is present in such looped complexes. Two of these enhancers also form complexes with a 3' boundary sequence that has CTCF sites. In addition, the 5' region of the active transcription unit exhibits a continuum of interactions with downstream chromatin segments. All of these interactions are B cell specific. Previous studies have shown that allelic exclusion of the mouse Igkappa locus occurs by the combination of monoallelic silencing and a low level of monoallelic activation for rearrangement combined with a negative feedback loop blocking additional functional rearrangements. Using yeast artificial chromosome-based single-copy isotransgenic mice, we have identified a cis-acting element that negatively regulates rearrangement in this locus, specifically in B cells. The element resides in the V-J intervening sequence, and is termed ***Sis*** (silencer in the intervening sequence). ***Sis*** specifies the targeting of germline Igkappa transgenes in B cells to centromeric heterochromatin and their association with Ikaros, a repressor protein that also co-localizes with centromeric heterochromatin. Significantly, these are hallmarks of silenced endogenous germline Igkappa genes in B cells. These results provide new insights into the molecular mechanisms of allelic exclusion.

752

**Genome Organizer SATB1 in Breast Carcinogenesis**

H. J. Han, T. Kohwi-Shigematsu; Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

It is largely unknown how chromatin is packaged in eukaryotic cells so that numerous genes are properly regulated in a cell-type and stage-specific manner. Our findings on SATB1 function introduced a concept that a single protein can control numerous genes as a genome organizer. SATB1 forms a unique intranuclear architecture which provides docking sites for specialized DNA sequences, enzymes that modify chromatin structure and protein factors necessary for gene expression (1,2). Thus SATB1 determines the local chromatin structure so as to confer a specific pattern of gene expression. Until now, most studies from our group on SATB1 focused on T cell development because SATB1 is predominantly expressed in thymocytes and activated T cells. Recently, we found SATB1 is also expressed in metastatic but not in non-metastatic breast cancer cell lines, and

primarily in aggressive human breast tumor specimens. Interestingly, in metastatic breast cancer cells SATB1 specifically targets a totally different set of genes than in thymocytes. Those genes regulated by SATB1 in breast cancer cells have important roles in breast cancer development and/or progression. We will describe the biological role of SATB1 in breast cancer progression as was analyzed using RNAi techniques. Our new data show that in breast cancer cells, SATB1 acts as a key player in re-organization of the genome that switches the pattern of gene expression such that the cells acquire metastatic properties. These findings indicate that changes in higher-order packing of chromatin is crucial for progression of breast tumorigenesis. References: Yasui, D., Miyano, M., Varga-Weisz, P. and Kohwi-Shigematsu, T. (2002). SATB1 targets chromatin remodelling to regulate genes over long distances. Nature. 419:641-645. Cai, S., Han, H., and Kohwi-Shigematsu, T. (2003). Tissue-specific nuclear architecture and gene function regulated by SATB1. Nature Genetics (article), 34:42-51.

## Coordination of Cytoskeletal Networks (753-758)

753

### Control of the Cytokinetic Rho Activity Zone by Gefs and Gaps

A. Miller, A. Clark, W. Bement; University of Wisconsin, Madison, Madison, WI

Cytokinesis is the final stage of cell division where one cell is separated into two. Classic experiments demonstrated that the spindle microtubules provide a signal that specifies the position where the actomyosin-based contractile ring should form. The nature of this signal and how it is delivered to the proper location remain mysterious, although emerging evidence implicates the small GTPase RhoA. We recently showed that a precisely-bounded, microtubule-dependent zone of high RhoA activity forms during cytokinesis in echinoderms and in Xenopus embryos. Importantly, these RhoA activity zones appear before furrowing begins and predict the location where the cleavage furrow will form. Moreover, recent work from other labs has shown that Ect2 (a RhoA GEF also known as Pbl), MgcRacGAP (a RhoA GAP also known as Cyk-4 or RacGAP50c), and MKLP (a kinesin also known as Zen-4, Pavarotti, or CHO1) are localized at the furrow and are important regulators of cytokinesis. Taken together, these results suggest that microtubules may specify the position of the contractile ring by precisely localizing regulators of RhoA activity, which then direct formation of a localized RhoA activity zone. Therefore, we are examining the roles of the RhoA regulators Ect2 and MgcRacGAP in modulating the RhoA activity zone during cytokinesis in Xenopus embryos. We find that both Ect2 and MgcRacGAP are required for cytokinetic RhoA activity zone formation, organization, and dynamics, explaining the necessity of these proteins for cytokinesis.

754

### Anillin and the Septins Increase the Efficiency of the Contractile Ring

A. S. Maddox, A. Desai, K. Oegema; Cell and Molecular Medicine, LICR / UCSD, La Jolla, CA

Constriction of the contractile ring changes the shape of the cell to facilitate cell division. We have developed live, fluorescence-based assays to analyze the roles of several structural components of the contractile ring in the *C. elegans* embryo. In addition to filamentous actin and myosin II, the septins form membrane-associated filaments that concentrate in the contractile ring. Anillin, another contractile ring component, interacts with all three cortical filament systems. Anillin is required to recruit the septins to the contractile ring, but localizes independently of myosin II and its activator, Rho-kinase. RNAi mediated depletion of myosin II completely inhibits furrow ingression. In contrast, depletion of either the septins or anillin results in only a slight late defect in the kinetics of furrow closure, but initiation and the majority of ingression are normal, and cytokinesis completes successfully. Interestingly, levels of equatorial myosin II are much higher throughout cytokinesis in anillin and septin depleted embryos than in control embryos. This observation raised the possibility that anillin and the septins decrease the amount of myosin II that is required for successful ingression. To test this idea, we utilized a sensitized condition in which the ability to activate myosin II is compromised. Rho-kinase contributes to myosin activation and recruitment in many systems. In *C. elegans*, depletion of Rho-kinase delays furrow initiation and slows ingression, but cytokinesis completes successfully. However, when anillin or the septins were depleted in conjunction with Rho-kinase, there was a synergistic defect in furrow ingression and cytokinesis failed. Two possible explanations for these results are: (1) anillin/septins increase the efficiency of the contractile machinery so that less myosin II is required, or (2) anillin/septins change the properties of the cortex, perhaps by reducing its rigidity, reducing the requirement for contractility.

755

### Drosophila Thrombospondin Promotes Muscle-specific Interaction with Tendon Cells

T. Volk, A. Subramanian; Molecular Genetics, Weizmann Institute, Rehovot, Israel

The formation of functional contractile system during embryonic development requires a perfect match between somatic muscles and their corresponding tendon cells. Migrating myotubes are guided to extend their leading edge towards tendon cells, born in the ectoderm germ layer. Once reaching the tendon cell, an EgfR-dependent signaling is triggered by the muscle, leading to elevation of the levels of the tendon-specific EGR-transcription factor Stripe. Stripe elevation induces tendon cell maturation and triggers the formation of muscle-tendon junction. By performing a microarray screen for stripe down-stream genes, we identified Drosophila Thrombospondin (Tsp). Thrombospondin is a tendon-specific extracellular matrix component. We have produced tsp mutants in which the conserved C-terminal domain of Tsp is deleted. This mutation is embryonic lethal. In embryos homozygous for tsp the somatic muscle pattern is disrupted; some muscles round up, and other muscles do not interact with tendon cells, and instead form ectopic junctions with neighboring muscles. Furthermore, the accumulation of the cytoplasmic protein Talin at the muscle-tendon junctions, which is indicative of the activation of Integrin-mediated signaling, is significantly reduced. Ectopic expression of Tsp in the entire embryonic ectoderm disrupts muscle patterning and forces the muscles to interact with non-tendon ectodermal cells. The activity, and ability to rescue the tsp mutant phenotype by expressing truncated Tsp proteins lacking either the N-terminal or C-terminal domains in tendon cells, is currently tested. These studies suggest that while guidance of the migrating muscles is performed by secreted proteins such as Slit, and possibly Netrin, Tsp function is necessary to signal the approaching muscle to specifically interact with the tendon cell, rather than with neighboring muscles. Thus, Tsp activity in Drosophila is reminiscent of the recently reported synapse-promoting activity of this gene product in the CNS.

756

### Abelson Kinase Coordinates Apical Constriction and Rhogef2 Localization During Drosophila Morphogenesis

D. T. Fox, S. L. Rogers, M. Peifer; Biology, University of North Carolina, Chapel Hill, NC

During animal development, coordinated changes in cell shape and position mold the body plan. As the actin cytoskeleton provides the structural basis for this morphogenesis, it is crucial to identify the function of actin regulatory proteins, as well as their interplay with one another. The *Drosophila* non-receptor tyrosine kinase Abl regulates actin structure in both epithelial cells and CNS axons by antagonizing Ena, the single fly Ena/VASP family member. To understand Abl's role during epithelial morphogenesis, we generated embryos lacking maternal and zygotic Abl. We find *abl* mutant embryos have specific defects in cell shape change during gastrulation. During wild-type gastrulation, cells of the presumptive mesoderm undergo a coordinated apical constriction, which leads to their internalization in a structure known as the ventral furrow. Using both live and fixed confocal imaging, we find that in *abl* mutants the presumptive mesodermal cells fail to constrict in a coordinated manner. As a result, their internalization is asynchronous, leading to aberrant ventral furrow. Consistent with a role in coordinating apical cell shape change, we find that Abl::GFP accumulates apically in constricting cells of the ventral furrow. Further, genetic interaction analysis suggests that Abl's role in this process includes negative regulation of Ena. As previous work in both fly embryos and S2 cells has established a role for the Rho activator RhoGEF2 and Myosin in the regulation of cell constriction, we examined their localization in *abl* mutants. We find that loss of Abl disrupts RhoGEF2 localization prior to apical constriction, but not Myosin localization. This observation has led us to use both fly genetics and an S2 cell model of cell constriction to test how Abl/Ena act in parallel or in concert with previously identified RhoGEF2 regulators to coordinate apical morphogenesis in *Drosophila*.

757

### Bipolar Spindle Assembly on Linear Chromatin Structures

J. Gaetz,[1] Z. Gueroui,[2] A. Libchaber,[2] T. M. Kapoor[1]; [1]Chemistry and Cell Biology, The Rockefeller University, New York, NY, [2]Experimental Condensed Matter Physics, The Rockefeller University, New York, NY

In recent years, much experimental evidence has demonstrated that positive signals generated by chromatin can assemble mitotic spindles in the absence of centrosomes. These signals contribute to the nucleation, stabilization and organization of microtubules and may determine the shape and size of the mitotic spindle. How the spatial distribution of the chromosomal signals influences cytoskeletal organization has not been directly tested. We have developed an experimental system that can be used to control the shape of chromatin, and therefore the positive signal, during spindle assembly. In cell-free extracts prepared from *Xenopus* oocytes, DNA-coated paramagnetic beads were patterned, using electromagnetic fields, into linear structures ranging from 6 to 100 μm in length and constant width. After packaging of DNA into chromatin in the extract, these chromatin bead "strings" were capable of generating sufficient positive signal to induce spindle assembly. Here we present new data examining, in detail, the effect of altered chromatin signaling patterns on the assembly of the bipolar spindle. Experiments using chemical and protein-based inhibitors of spindle pole organization have uncovered opposing roles for signals from chromatin and spindle poles in regulating the proper size and shape of the bipolar spindle. The roles of cytoskeletal motor proteins and non-motor microtubule-associated proteins are examined. Our findings have implications for mechanisms of regulation of the cell division apparatus, as well as other dynamic intracellular structures.

758

### Two Spiral Structures Control Cell Shape in *Caulobacter crescentus*

N. A. Dye,[1] J. A. Theriot,[1] L. Shapiro,[2] Z. Gitai[3]; [1]Biochemistry, Stanford University, Stanford, CA, [2]Developmental Biology, Stanford University, Stanford, CA, [3]Molecular Biology, Princeton University, Princeton, NJ

Mutations in the bacterial actin homolog, *mreB*, confer a spherical morphology to the normally rod-shaped bacteria *E.coli*, *B.subtilis*, and *C.crescentus*. To learn more about the mechanism by which the actin cytoskeleton contributes to shape determination in bacteria, we took a cell biological approach to study two genes coexpressed with *mreB* in *Caulobacter crescentus*. *mreC* lies immediately downstream of *mreB*. When mRFP1 was fused to the putative periplasmic C-terminus of MreC, we observed a punctate, helical pattern. Upon labeling both MreB and MreC in the same strain we found, amazingly, that these two helices do not overlap. A deletion of *mreC* could not be obtained, indicating that the gene is essential in *Caulobacter*. When MreC was depleted, *Caulobacter* became lemon-shaped, mimicking the MreB depletion phenotype. MreB, however, was still able to form dynamic structures in MreC-depleted cells, indicating that MreC is not required to localize MreB. Interestingly, MreC did not require MreB for proper localization: in the presence of the MreB-inhibitor, A22, MreC still formed spirals. The gene *pbp2*, encoding a peptidoglycan transpeptidase, is also found in the *mre* operon in *Caulobacter*. When fused to GFP, Pbp2 was localized in a helical pattern overlapping with the MreC but not MreB spirals. Surprisingly, perturbing either MreB (with A22) or MreC (with depletion) caused GFP-Pbp2 to mislocalize to a midcell position, potentially providing a molecular explanation for the shape defects observed after depletion for either MreB or MreC. Depletion of FtsZ prevents this mislocalization of GFP-Pbp2 and partially rescues the cell shape defect in A22. Together our results demonstrate the existence of two separate and independent protein spirals in *Caulobacter*. These spirals, one cytoplasmic and one periplasmic, have opposing roles in regulating the localization of a transpeptidase and consequently cell shape.

## Nuclear Compartments (759-764)

759

### Analysis of Dynamic Changes in the Nucleolar Proteome and PP1 Complexes using Time-Lapse Proteomics and Time-Lapse Fluorescence Microscopy

A. I. Lamond,[1] Y. W. Lam,[1] L. Trinkle-Mulcahy,[1] J. Andersen,[2] M. Mann[2]; [1]Gene Regulation & Expression, University of Dundee, Dundee, United Kingdom, [2]Biochemistry & Molecular Biology, University of Southern Denmark, Odense, Denmark

The cell nucleus is a highly compartmentalized organelle and many nuclear proteins, including splicing factors, are organized into distinct classes of nuclear bodies, such as nucleoli, PML bodies, Cajal bodies, speckles and paraspeckles. We are studying the functional organization of mammalian cell nuclei with the aim of understanding how specific nuclear compartments are assembled and how individual protein and RNA-protein complexes are targeted to these compartments and are able to move between them (see www.LamondLab.com). We are using a

combination of quantitative proteomic methods, involving metabolic labeling of proteins with amino acids containing heavy isotopes $^{13}C$ and $^{15}N$, and quantitative in vivo imaging techniques. Experiments using multiplexed heavy isotope labeling (SILAC) and quantitative mass spectrometry have identified changes in the protein composition of the nucleolus under different metabolic conditions. Parallel studies using quantitative digital fluorescence microscopy confirm the dynamic behaviour of nucleolar proteins. A detailed description of the nucleolar proteome and the SILAC data is available in a searchable online database (see http://lamondlab.com/nopdb/). We have also used the SILAC approach in combination with time-lapse fluorescence imaging to characterize the specific interactions of targeting proteins with isoforms of protein phosphatase 1 (PP1). We have identified a novel PP1 binding factor, termed 'RePO-Man', that selectively targets a pool of PP1gamma to chromatin during mitosis at the metaphase-anaphase transition. This work offers a general approach for characterizing dynamic changes in the composition of either organelles or multi-protein complexes.

760

**Drosophila Cajal Body Components**

J. Liu,[1] C. Murphy,[1] M. Buszczak,[1] S. Clatterbuck,[2] R. Goodman,[2] J. Gall[1]; [1]Department of Embryology, Carnegie Institution of Washington, Baltimore, MD, [2]Department of Biology, Johns Hopkins University, Baltimore, MD

Cajal bodies (CBs) are intranuclear organelles that contain many components involved in transcription and processing of cellular RNA. Identification of CBs relies heavily on specific marker proteins and small RNAs, especially the protein p80-coilin. Because coilin has not been identified in *Drosophila*, we examined the U7 snRNP, which is limited almost exclusively to CBs in *Xenopus* oocytes and HeLa cells. The sequences of *Drosophila* U7 snRNA and two proteins specific for the U7 snRNP, Lsm10 and Lsm11, have been described. We produced transgenic lines of *Drosophila* that express Lsm11-GFP. We examined early embryonic blastoderm, third instar larval brains and salivary glands, and adult ovaries. The great majority of cells exhibit a single bright focus of Lsm11-GFP in their nuclei, except for nurse cell nuclei, which have multiple foci. Fluorescent in situ hybridization (FISH) demonstrated the presence of U7 snRNA in all these foci. Furthermore, FISH of a histone H4 probe showed that histone genes are colocalized with Lsm11-GFP and U7 snRNA. In *Xenopus* oocytes and HeLa cells some CBs are usually attached to the chromosomes at the histone gene loci; in *Drosophila* the association appears to be invariant. We examined two other typical CB components, U2 snRNA and U85 scaRNA. U85 was concentrated in a focus distinct from the U7 focus. Some of the U2 colocalized with the U85 focus and the rest was more diffusely spread throughout the nucleus. We will examine additional CB components in *Drosophila* to determine how their overall distribution compares with that in mammalian and *Xenopus* nuclei. In a mouse strain deficient for coilin, CB components that are typically contained in a single body are distributed into three separate nuclear bodies. An interesting possibility is that the lack of coilin in *Drosophila* results in a similar redistribution of CB components.

761

**Neuromuscular Development and snRNPs: A Fly Model for Spinal Muscular Atrophy**

T. K. Rajendra, G. Gonsalvez, H. K. Salz, G. A. Matera; Genetics, Case Western Reserve University, Cleveland, OH

Small nuclear ribonucleoproteins (snRNPs) of the Sm-class are essential pre-mRNA splicing factors. In metazoans, pre-snRNA transcripts are exported to the cytoplasm by the export adaptor, PHAX. Cytoplasmic assembly into stable Sm-core particles is mediated by the survival motor neurons (SMN) protein complex. Following additional cytoplasmic remodeling steps, the RNPs are imported into the nucleus, where they undergo further maturation and ultimately assemble into spliceosomes. Thus, the biogenesis of Sm snRNPs is a multi-step process that takes place in distinct subcellular compartments. Mutations in the human *SMN1* gene result in the neurogenetic disease, Spinal Muscular Atrophy (SMA). Although *SMN* mutations are associated with defects in formation of Sm-core RNPs *in vitro*, the link to the etiology of SMA *in vivo* is unclear. To address this question, we have developed a model system in *Drosophila* to study snRNP biogenesis. Hypomorphic mutations in the fruitfly *smn* and *smd2* genes and complete loss-of-function alleles of *phax* display an interesting phenotype that resembles SMA in several details. Both genes are essential for organismal viability and fertility, however, a fraction of the expected homozygous mutants escape the embryonic and larval lethality and survive to adulthood. These 'escapers' are sterile and unable to jump or fly, with defects in flight muscle myofibril organization. Homozygous *phax* mutants show an observable decrease in the levels of U1 and U2 snRNAs, but not in U3 or U6 RNAs. Collectively, these findings indicate a critical role for snRNPs during neuromuscular development and provide a strong connection between snRNP biogenesis and the molecular etiology of SMA.

762

**Splicing Independent Recruitment of snRNPs to Active Transcriptional Units**

S. B. Patel,[1] M. Bellini[2]; [1]Biochemistry and College of Medicine, University of Illinois at Urbana-Champaign, urbana, IL, [2]Cell & Developmental Biology, University of Illinois at Urbana-Champaign, urbana, IL

Work over the past decade has shown that the splicing of pre-mRNAs begins co-transcriptionally. However, the mechanisms that regulate the association of the spliceosomal small nuclear ribonucleoprotein particles (snRNPs) to transcriptional units remain elusive. In amphibian oocytes, RNA polymerase II transcription sites correspond to the extended lateral loops of the lampbrush chromosomes (LBCs), which are readily observable by light microscopy. Here, we show for the first time that injected fluorescently-labeled U1, U2, and U5 (a representative member of the U4/U6.U5 tri-snRNP) snRNAs do target the chromosomal loops in addition to their previously reported association with other nuclear compartments. As we were able to detect synthetic snRNAs on the LBC loops, we were curious to see whether a mutant form of U1 snRNA (ΔU1) that lacks the region required for 5'-splice site (5'-SS) recognition, and hence cannot participate in splicing, is still targeted to chromosomal loops. Surprisingly, we observed an association of ΔU1 with chromosomal loops, indicating that 5'-SS recognition is not necessary for recruiting U1 to transcription sites. This result prompted us to ask whether the snRNPs would still localize to LBC loops in absence of U2, which was previously shown to be essential for the loading of U1 and U5 onto pre-mRNA. Interestingly, in oocytes depleted of U2 snRNAs, and thus deficient in splicing, we observed that the chromosomal targeting of U1 and U5 was not affected. Taken together, these data indicate that the recruitment of snRNPs to active transcriptional units does not depend on splicing and support a two-step "recruitment-loading" model. In this model, snRNPs are first recruited to transcription sites and then loaded onto their pre-mRNA substrate.

763

**Live Cell Dynamics of PNC Components**

K. Kopp, S. Huang; Cell and Molecular Biology, Northwestern University, Chicago, IL

The perinucleolar compartment (PNC) is a sub-nuclear structure that notably localizes to the periphery of the nucleolus. The PNC is found predominantly in transformed cells both *in vitro* and *in vivo*. PNCs are highly enriched with newly synthesized RNA polymerase III transcripts and RNA binding proteins. The structural integrity of the PNC is dependent upon the transcription of these RNAs and a critical level of the polypyrimidine tract binding protein (PTB), as assayed by the localization of other PNC associated proteins. These and other observations suggest a model in which the PNC is a dynamic, functional organelle that forms under specific physiological conditions favoring cellular transformation and might be involved in the metabolism of RNA polymerase III transcripts. In order to further understand the functional role of the PNC in the metabolism of the small RNAs accumulated within, we have made an array of fusion proteins between photo-activatable GFP and a group of RNA binding proteins known to be concentrated in the PNC. Using live cell imaging, we have been able to trace the movement of proteins after leaving the PNC. Additional results will be presented.

764

**Regulation of Ubiquitin Ligase Dynamics by the Nucleolus**

K. Mekhail,[1] M. Khacho,[1] A. Carrigan,[2] R. Hache,[2] L. Gunaratnam,[1] S. Lee[1]; [1]Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, [2]Ottawa Health Research Institute, University of Ottawa, Ottawa, ON, Canada

Cellular pathways relay information through dynamic protein interactions. We have assessed the kinetic properties of the MDM2 and VHL ubiquitin ligases in living cells under physiological conditions that alter the stability of their respective p53 and HIF substrates. Photobleaching experiments reveal that MDM2 and VHL are highly mobile proteins in settings where their substrates are efficiently degraded. The nucleolar architecture converts MDM2 and VHL to a static state in response to regulatory cues that are associated with substrate stability. Following signal termination, the nucleolus is able to rapidly release these proteins from static detention, thereby restoring their high mobility profiles. A protein surface region of VHL's β-sheet domain was identified as a discrete [H+]-responsive nucleolar detention signal ($NoDS^{H+}$) that targets the VHL/Cullin-2 ubiquitin ligase complex to nucleoli in response to physiological fluctuations in environmental pH. Data shown here provide the first evidence that cells have evolved a mechanism to regulate molecular networks by reversibly switching proteins between a mobile and static state.

## Pathogens Co-opting Host Cell Functions (765-770)

765

**Clustering of $EspF_U$ Beneath the Plasma Membrane of Mammalian Cells is Sufficient to Trigger Localized Actin Assembly**

K. G. Campellone, D. Robbins, J. M. Leong; Molecular Genetics and Microbiology, UMass Medical School, Worcester, MA

Enterohemorrhagic *E. coli* (EHEC) are extracellular pathogens that utilize a specialized translocation system to deliver effector proteins into mammalian cells and reorganize the cytoskeleton into actin-rich pedestals. One effector that is essential for actin pedestal formation is Tir, a protein that inserts into the mammalian plasma membrane and acts as a receptor for the bacterial surface adhesin, intimin. EHEC additionally translocates $EspF_U$ (also known as TccP), a second critical effector of actin polymerization that mediates recruitment of the actin assembly machinery to Tir. The crucial signaling function of $EspF_U$ resides in its proline-rich domain, which contains 6 nearly-identical 47-residue peptide repeats. This region of $EspF_U$ can bind to N-WASP, a key activator of the Arp2/3 actin-nucleating complex. To define the minimal signaling requirements for actin pedestal formation by EHEC, Tir and $EspF_U$ were expressed directly in mammalian cells. Clustering of a membrane-targeted Tir derivative in the presence of $EspF_U$ was sufficient to trigger localized actin assembly, indicating that $EspF_U$ and Tir are the only two EHEC effectors necessary for pedestal formation. While a 12-residue C-terminal peptide was revealed to be the only cytoplasmic sequence of Tir necessary for signaling, a single 47-residue $EspF_U$ repeat was determined to be sufficient for interacting with N-WASP and stimulating localized actin assembly, although two repeats promoted pedestal formation more efficiently. The C-terminus of Tir serves merely to cluster $EspF_U$, because a Tir derivative in which this domain was replaced with the proline-rich region of $EspF_U$ was still functional for pedestal formation. EHEC-mediated actin assembly is more complicated than simple recruitment of N-WASP, however, because $EspF_U$ appears to interact with multiple regulators of actin polymerization.

766

**Dynamics of Polyomavirus Binding, Endocytosis and Intracellular Trafficking**

A. E. Smith,[1] H. Ewers,[1] J. Kartenbeck,[2] H. Lilie,[3] A. Helenius[1]; [1]Institute of Biochemistry, Swiss Federal Institute of Technology, Zurich, Switzerland, [2]Deutsches Krebsforschungszentrum, Heidelberg, Germany, [3]Martin-Luther-University, Halle, Germany

For productive cell entry and infection, animal viruses make use of a variety of alternative endocytic pathways. We have followed viruses as they attach to the surface of cells, move laterally along the plasma membrane, enter via endocytic vesicles and traffic in the cytoplasm. Our analysis of polyomavirus entry and infection revealed a novel, poorly characterized pathway for cellular endoyctosis and intracellular trafficking. It is initiated by high-affinity, stable membrane attachment requiring ganglioside receptors. Upon binding, individual Py virus-like particles (VLPs) generally exihibited rapid random motion, followed by actin filament-dependent confinement, as revealed by single fluorescent particle tracking performed on cells or planer supported lipid bilayers. Real-time fluorescence microscopy in combination with thin section electron microscopy, biochemical and biophysical assays revealed that for endocytosis, tyrosine kinase activity was induced, and an uncoated, cholesterol dependent, primary endocytic vesicle was formed. Virus entry was rapid, and an Arf6-, dynamin 2-, and actin-independent process. The virus-marked, pH-neutral, intracellular vesicle was initially independent of markers for classical endosomal pathway (Rab GTPases, fluid phase, transferrin, Semliki Forest virus), or of caveolin-1, and later merged with intracellular caveosomes. Overall, viruses are proving valuable tools to address the complexities of endocytic membrane trafficking and lipid domain organization.

767

**Listeria Hijacks the Clathrin-dependent Endocytic Machinery to Invade Mammalian Cells**

E. Veiga, P. Cossart; UIBC, Institut Pasteur, Paris, France

The bacterial pathogen Listeria monocytogenes uses the surface protein InlB to invade a variety of cell types. The interaction of InlB with the hepatocyte growth factor receptor, Met, is critical for infection. Here we show that purified InlB induces the Cbl-dependent mono-ubiquitination and endocytosis of Met. We then demonstrate that the bacterium exploits the clathrin-dependent endocytosis machinery to invade mammalian cells. First, we show that Listeria colocalizes with Met, EEA1, the ubiquitin ligase Cbl, clathrin and dynamin during entry. Then we dissect the role played by different proteins of the endocytic machinery during Listeria infection. Over-expression or down-regulation of Cbl respectively increases or decreases bacterial invasion. Furthermore, RNAi-mediated knock-down of major components of the endocytic machinery e.g. clathrin, dynamin, eps15, Grb2, CIN85, CD2AP, cortactin and Hrs, inhibit bacterial entry, establishing for the first time that the endocytic machinery is critical for the bacterial internalization process.

768

**Mechanisms of Sequestration of Host Lysosomes in the Unique Intracellular Vacuole Containing *Toxoplasma***

I. Coppens,[1] M. Pypaert,[2] K. A. Joiner[3]; [1]Molecular Microbiology and Immunolgy, Johns Hopkins School of Public Health, Baltimore, MD, [2]Cell Biology, Yale University, New Haven, CT, [3]Internal Medicine, Yale University, New Haven, CT

*Toxoplasma gondii* is adapted to thrive in a unique self-made niche, i.e. the parasitophorous vacuole (PV) within the cytoplasm of a large variety of mammalian cells. The PV of *Toxoplasma* is a nonfusogenic compartment, which segregates the parasites from the host cell vesicular transport system. We identified a novel strategy for nutrient acquisition by intravacuolar *Toxoplasma*. The PV accumulates material coming from the host cell via the exploitation of the host endo-lysosomal system. Videofluorescence and electron microscopic studies reveal that *T. gondii* actively recruits host microtubules around the PV, resulting in selective attraction of endo-lysosomes to the PV membrane. Interference with the function of host microtubules or lysosomes abolishes the association of PV-endocytic structures. Polymerization of host microtubules causes microtubule-based invaginations of the PV membrane. These tubular extensions serve as conduits for the delivery of host endo-lysosomes within the PV. Such conduits are decorated by transverse coated striations on their surface, reminiscent of the collars induced by dynamin. Indeed, the coat acts like a garrote that sequesters host endocytic organelles within the vacuolar space. Among parasite proteins secreted in the PV, GRA7 has the property to bind lipids, constrict spherical liposomes into branched tubules and form homo-oligomers in a liposome-stimulated manner via C-terminal hydrophobic residues. This leads to the assumption that GRA7 may constitute one major molecule forming the electron-dense coat constricting the tubular extensions of the PVM. In conclusion, these data define a new process allowing the *T. gondii* intimate and concentrated access to a diverse range of low molecular weight components produced by the endo-lysosomal system. More generally, they identify a new mechanism for unidirectional transport and sequestration of host organelles.

769

**Vaccinia Virus induced Cell Motility Involves Inhibition of RhoA Signalling**

F. Valderrama,[1] J. Cordeiro,[1] S. Schleich,[1] F. Frischknecht,[2] M. Way[1]; [1]Cell Motility Group, Cancer Research UK, London Research Institute, London, United Kingdom, [2]Department of Parasitology, Heidelberg University School of Medicine, Heidelberg, Germany

The spatial and temporal regulation of cell adhesion and motility is essential during development and throughout the lifetime of multi-cellular organisms. Deregulation of these two fundamental cellular processes frequently occurs during pathological situations and is an important factor contributing to tumour cell metastasis. Dramatic changes in cell migration and adhesion, as well as loss of contact inhibition, are also observed during viral infections including that of vaccinia virus. Using a combination of genetic and biochemical approaches we have identified a viral protein that is critically required for vaccinia-induced cell motility. This vaccinia protein, which is conserved in the genomes of orthopox viruses, binds directly to RhoA to inhibit interactions with its downstream effectors, ROCK and mDia1. Molecular ablation of this viral protein using siRNA techniques results in a loss of vaccinia-induced cell motility as well as dramatic inhibition of viral morphogenesis. Based on our observations, we suggest that modulation of RhoA signaling during the vaccinia life cycle is critically required for the continued spread of infection.

770

**Infectious Contact- Retroviral Transmission via Filopodia**

N. M. Sherer, M. J. Lehmann, P. D. Uchil, W. Mothes; Section of Microbial Pathogenesis, Yale University, New Haven, CT

During infection, enveloped viruses engage their cellular receptors and then move laterally along filopodia towards the cell body where they undergo entry (Lehmann et al, 2005. J Cell Bio 170:317-25). This transport is driven by the underlying retrograde F-actin flow and is dependent on myosin II. Like classical ligands such as EGF, viruses are transported along the cell surface to reach endocytic hot spots at the cell body prior to envelope fusion or endocytosis. Here, we show using live cell imaging that during virus assembly, in striking contrast to entry, retroviruses such as murine leukemia virus (MLV) can assemble and bud from the tips of filopodia, demonstrating that the peripheral actin cytoskeleton of infected cells supports virus movement in the opposite direction, away from the cell. Thus, infected cells apparently present retroviruses at the tips of their outermost protrusions. Co-culture experiments revealed viruses passed on from the protrusions of infected cells to filopodia of uninfected cells where they engaged rapid retrograde flow towards the cell body. These experiments demonstrate virus transmission via short contacts between filopodia rather then tight cell-cell interactions establishing an "infectious synapse" (McDonald et al, 2003. Science 300:1295-7). We predict that the contrasting tendencies of MLV trafficking seen for producer vs. target cell reflects the relative affinities of the ligand-receptor interactions involved. As such, viruses are again shown to be exploiting general pathways of cell signaling and intercellular communication.

## Regulating Intercellular Junctions (771-776)

771

**Cooperative Roles of Integrin and Nectin in the Formation of Cadherin-based Adherens Junctions**

H. Ogita, Y. Sakamoto, T. Hirota, Y. Takai; Molecular Biology & Biochemistry, Osaka University, Osaka, Japan

Cell-matrix adhesion molecule integrin has been implicated in the formation and maintenance of cell-cell junctions. Immunoglobulin-like cell-cell adhesion molecule (CAM) nectin homophilically or heterophilically *trans*-interacts with each other in a $Ca^{2+}$-independent manner to form cell-cell

contacts, where $Ca^{2+}$-dependent CAM cadherin is recruited, eventually causing formation of adherens junctions (AJs). In addition, the *trans*-interaction of nectin induces activation of Cdc42 and Rac small G proteins through c-Src and forms filopodia and lamellipodia, respectively, facilitating the formation of AJs. However, the mechanism by which integrin is involved in the formation of AJs remains to be elucidated. We examined here the physical and functional relationship between integrin $\alpha_v\beta_3$ and nectin in the formation of AJs in NIH3T3 cells. Integrin $\alpha_v\beta_3$ co-localized and associated with nectin at the nectin-based cell-cell contact sites. Furthermore, integrin $\alpha_v\beta_3$ associated with nectin at the earlier stage of the formation of AJs had the extended conformation determined by WOW-1 Fab. Then, after the establishment of AJs, the extended conformation of integrin $\alpha_v\beta_3$ diminished at AJs, although the signal for integrin $\alpha_v\beta_3$ determined by LM609 mAb was observed there together with that for N-cadherin, suggesting that the bent conformation of integrin $\alpha_v\beta_3$ exists at established AJs. On the other hand, the nectin-induced, c-Src-mediated activation of Cdc42 and Rac required the activation of integrin $\alpha_v\beta_3$. Focal adhesion kinase, which relays the integrin-mediated outside-in signals to the intracellular signaling molecules, was also necessary for the nectin-induced signaling and was involved in the association of integrin $\alpha_v\beta_3$ with nectin. Thus, integrin $\alpha_v\beta_3$ and nectin play cooperative roles in the formation of cadherin-based AJs.

772

**Non-static Linkage between Cadherin Complex and Actin Cytoskeleton**

S. Yamada, W. J. Nelson; Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA

Cell-cell adhesion is thought to be initiated through *trans* interactions between cadherin extracellular domains, and strengthened by linkage of cadherins to the actin cytoskeleton. However, we reported that α-catenin does not bind simultaneously to both E-cadherin/β-catenin and actin filaments. Upon initial cell-cell adhesion, the circumferential actin network near the initial contact site is disrupted, and lamellipodial activity, which is driven by Arp2/3 and initially active at cell-cell contacts, is dampened around maturing contacts. Arp2/3-mediated lamellipodia activity becomes restricted to the edges of cell-cell contact expansion where the circumferential actin cable terminates. Furthermore, paxillin, a focal adhesion protein, also co-localizes at this molecular complex suggesting a role of integrin adhesion in actin organization at this site. In vitro, α-catenin can suppress actin polymerization induced by the Arp2/3 complex, thus α-catenin may play an essential role in the actin reorganization during the initial stage of cell-cell adhesion. We will discuss implications of non-static linkage between cadherin-catenin complex and actin filaments during these stages of cell-cell contact formation.

773

**Preserving Junctions: Myosin 2 is a Key Rho kinase Target Necessary for the Local Concentration of E-cadherin at Cell-cell Contacts**

A. M. Shewan,[1] M. Maddugoda,[1] A. Kraemer,[1] S. J. Stehbens,[1] S. Verma,[1] E. M. Kovacs,[2] A. S. Yap[1]; [1]University of Queensland, Institute for Molecular Bioscience, Brisbane, Australia, [2]University of Queensland, School for Biomedical Science, Brisbane, Australia

Classical cadherin adhesion molecules are key determinants of tissue morphogenesis. Cadherins accumulate at cell-cell contacts as a characteristic response to homophilic ligation and this is necessary for junction assembly and tissue cohesion. The ability of cells to concentrate cadherins at contacts is a surprisingly complex process: it requires productive binding of the ectodomain to cooperate with cytoplasmic determinants, many of which remain poorly characterised. We recently identified Myosin 2 as one such cytoplasmic factor that determines whether E-cadherin can concentrate at adhesive contacts. Myosin 2 accumulated at cell-cell contacts in a cadherin-dependent manner; indeed adhesion to recombinant cadherin ligands was sufficient both to recruit and to activate Myosin 2. Inhibition of Myosin 2 activity, either directly using blebbistatin or by inhibiting MLCK, significantly reduced cadherin accumulation at cell-cell contacts measured both by quantitative immunofluorescence microscopy and by FRAP. However, total and surface levels of E-cadherin were not altered, indicating that Myosin 2 critically controlled the ability of surface E-cadherin to concentrate regionally in cell-cell contacts. The impact of Myosin 2 inhibitors on cadherin accumulation was precisely mimicked by Y27632, an inhibitor of Rho kinase. Moreover, Rho kinase signaling was necessary for cadherin adhesion to recruit and activate Myosin 2. We conclude that Myosin 2 is a key effector of Rho kinase signalling that critically regulates the ability of cadherin to concentrate at contacts in response to homophilic ligation.

774

**P120-Catenin Regulates Clathrin-Dependent Endocytosis of VE-cadherin**

K. Xiao,[1] J. Garner,[1] K. M. Buckley,[2] P. A. Vincent,[3] C. M. Chiasson,[2] E. Dejana,[4] V. Faundez,[5] A. P. Kowalczyk[1]; [1]Dermatology, Emory University, Atlanta, GA, [2]Graduate Program in Biochemistry, Cell, and Developmental Biology, Emory University, Atlanta, GA, [3]The Center for Cardiovascular Science, Albany Medical College, Albany, NY, [4]FIRC Institute of Molecular Oncology and Department of Biomolecular and Biotechnological Sciences, University of Milan, Milan, Italy, [5]Cell Biology, Emory University, Atlanta, GA

VE-cadherin is an adhesion molecule critical to vascular barrier function and angiogenesis. VE-cadherin expression levels are regulated by p120 catenin, which prevents lysosomal degradation of cadherins by unknown mechanisms. To test whether the VE-cadherin cytoplasmic domain mediates endocytosis, and to elucidate the nature of the endocytic machinery involved, the VE-cadherin tail was fused to the IL-2 receptor extracellular domain (IL-2R). Internalization assays demonstrated that the VE-cadherin tail dramatically increased endocytosis of the IL-2R in a clathrin-dependent manner. Interestingly, p120 inhibited VE-cadherin endocytosis via a mechanism that required direct interactions between p120 and the VE-cadherin cytoplasmic tail. However, p120 did not inhibit transferrin internalization, demonstrating that p120 selectively regulates cadherin internalization rather than globally inhibiting clathrin-dependent endocytosis. Finally, cell surface labeling experiments in cells expressing GFP tagged p120 indicated that the VE-cadherin-p120 complex dissociates upon internalization. These results support a model in which the VE-cadherin tail mediates interactions with clathrin-dependent endocytic machinery, and that this endocytic processing is inhibited by p120 binding to the cadherin tail. These findings suggest a novel mechanism by which a cytoplasmic binding partner for a transmembrane receptor can serve as a selective plasma membrane retention signal, thereby modulating the availability of the protein for endo-lysosomal processing.

775

**Consequences of p120ctn Ablation in the Mouse Colon and Similarities to Human Inflammatory Bowel Disease**

W. G. Smalley,[1] P. E. Burnett,[2] M. A. Davis,[1] A. B. Reynolds[1]; [1]Cancer Biology, Vanderbilt University, Nashville, TN, [2]Division of Dermatology, Vanderbilt University, Nashville, TN

siRNA-mediated p120ctn (p120) ablation *in vitro* indicates that p120 regulates E-cadherin stability and cell-cell adhesion. To examine the effects of p120 ablation in an animal model, we have generated a conditional p120 knockout mouse using Cre-loxP technology. p120 ablation was targeted to the small intestine and colon by crossing our p120 floxed mice with Villin-Cre transgenic animals from Deborah Gumucio. Immunofluorescence staining of tissue sections reveal mosaic p120 knockout in approximately half of epithelial cells from the stomach to the anus, with no other tissues affected. p120 negative regions display significant E-cadherin downregulation and striking defects in epithelial morphology and adhesion. Damage and premature enterocyte shedding cause bleeding, diarrhea, dehydration, and failure to thrive. Death occurs between 11 and 23 days after birth due to excessive bleeding into the lumen and extraordinary drop in blood hematocrit. At the cell and tissue level, we observe striking mucosal hyperplasia in the colon and over 2 fold increases in proliferation rates for both intestinal and colonic epithelium. Interestingly, there is striking recruitment of neutrophils (but not macrophages) to p120 negative regions of the colon but not small intestine suggesting an early inflammatory response. p120 downregulation has been reported in human inflammatory bowel (IBD) diseases including Crohn's disease (75% of cases) and ulcerative colitis (100% of cases), the latter being a significant precondition for colon cancer. Preliminary comparisons of p120 downregulation in human IBD tissue to p120 ablation in the mouse colon reveal striking similarities at several levels, suggesting that p120 downregulation could be causally associated with this condition in humans.

776

### Microtubules Target Sites of Cell-Cell Contact and May Regulate Adhesion Dynamics

L. A. Ligon, E. L. F. Holzbaur; Physiology, University of Pennsylvania, Philadelphia, PA

The regulation of intercellular junction formation and stabilization is critical to maintain the balance between adhesion and migration, and failures in this regulation can lead to pathologies such as carcinogenesis and metastasis. The actin cytoskeleton has been shown to be an essential component of this regulation. Here we show that microtubules play a role in this regulation as well. Microtubules target epithelial adherens junctions, where they are transiently tethered at sites of cell-cell contact by a web of protein interactions that includes cytoplasmic dynein, PLAC-24 and β-catenin. These tethered microtubules appear to play a key role in the regulation of junction assembly. The number of microtubules targeting mature junctions is relatively small, but is significantly higher during junction assembly. Depolymerization of microtubules or disruption of microtubule dynamics inhibits junction formation. Cytoplasmic dynein is localized to initial points of contact between cells, and microtubules project directly to these dynein patches where they become tethered. Direct inhibition of cytoplasmic dynein function by microinjection of anti-dynein intermediate chain antibodies disrupts the tethering of microtubules and inhibits junction formation. These tethered microtubules may serve as tracks for directed cargo trafficking, and immunocytochemistry with junctional components such as α-catenin and p120catenin suggests that they are deposited proximal to tethered microtubules. However, these tethered microtubules may also serve as tracks for retrograde signalling to convey information about the state of the forming junction to the cell center. Together these data suggest that microtubules tethered at sites of cell-cell contact by cytoplasmic dynein are critical in the regulation the dynamics of intercellular adhesion. *Supported by NIH GM068591.*

## Signaling in 3D Environments (777-782)

777

### 3D Tissue Architecture and SMRT-dependent Death Resistance

C. Chatterjee,[1] M. Nuth,[1] J. Leight,[2] J. N. Lakins,[1] E. Bernhard,[3] V. M. Weaver[1]; [1]Institute of Medicine and Engineering/Pathology, University of Pennsylvania, Philadelphia, PA, [2]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA, [3]Department of Radiation Biology, University of Pennsylvania, Philadelphia, PA

Apoptosis resistance plays a critical role in tumor progression and treatment responsiveness. We have been studying how cell-ECM interactions modify survival of mammary tumors. We identified laminin-α6β4 integrin-NFκB as an important mechanism that can mediate apoptosis resistance in mammary tissues (Weaver et al., Cancer Cell 2002; Zahir et al., J Cell Biol 2003). We found that the efficacy of NFκB-dependent survival is dramatically enhanced when MECs are grown to form three-dimensional (3D) tissues. Basement membrane (BM)-ligated MECs in both 2D and 3D similarly permit NFκB activation and induction of anti-apoptotic genes (IAP-1, A20). Yet, NFκB activation in MECs in 2D but not in 3D significantly increases expression of multiple stress response genes (ATF2, IL8). Interestingly, we found that treatment with the histone deacetylase inhibitor TSA increases stress gene expression and renders 3D MEC structures apoptotically sensitive; consistent with micro-array data showing that 3D MEC structures repress expression of hundreds of stress-response genes, and our contention that epigenetics modulates NFκB-dependent apoptosis resistance of 3D tissues. Consistently, we found that apoptotically-resistant 3D non-malignant MEC structures and phenotypically-reverted tumors up-regulate expression of the chromatin repressors SMRT/NCoR (RT-PCR, immunoblot), and that knocking down SMRT/NCoR (siRNAs) renders 3D MEC structures highly sensitive to multiple apoptotic insults. Moreover, ectopic expression of SMRT permits tumors and even isolated tumor cells to resist γ-radiation (12Gy), immune-receptor (TRAIL) and chemotherapy (Taxol)-induced apoptosis, and SMRT knockdown up-regulates expression of multiple stress-response genes. These data indicate that 3D tissue architecture may enhance NFκB-dependent survival by repressing cell stress through modulation of epigenetic factors such as SMRT. Because SMRT activity and turnover are modulated by adhesion-dependent signaling phosphorylation (SAPK, ERK), and 3D MECs have blunted ERK and SAPK activity, we are exploring links between 3D, the stress response and SMRT-dependent apoptosis resistance. DOD W81XWH-05-1-330, CA078731.

778

### An Isoform-Specific Role for Akt1 in Inhibition of Epithelial-Mesenchymal Transition

H. Irie,[1,2] R. Pearline,[1] D. Grueneberg,[3] M. Hsia,[3] P. Ravichandran,[3] N. Kothari,[3] S. Natesan,[3] J. Brugge[1]; [1]Cell Biology, Harvard Medical School, Boston, MA, [2]Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, [3]Sanofi-Aventis Cambridge Genome Center, Boston, MA

Insulin-like growth factor-I receptor (IGF-IR) signaling has been implicated in the pathogenesis of breast and other human cancers. Akt/PKB kinases are activated downstream of IGF-IR and other growth factors, and regulate cell proliferation, survival and migration. However, recent evidence suggests that the three Akt isoforms contribute differentially to key cellular activities. Here we demonstrate that downregulating expression of two distinct Akt isoforms using short hairpin RNA vectors has dramatically distinct consequences on IGF-IR-induced phenotypic changes in both monolayer and three-dimensional (3D) basement membrane cultures of breast epithelial cells. Downregulation of Akt2 reverts

IGF-I induced morphological alterations in monolayer cultures, and the anti-apoptotic and hyperproliferative effects in 3D cultures. In contrast, Akt1 downregulation promoted neomorphic effects consistent with an epithelial-mesenchymal transition (EMT) with enhancement in migration, formation of invasive structures in 3D cultures, and expression of mesenchymal markers. The alterations induced by Akt1 downregulation were accompanied by enhancement of Erk/MAP kinase activity and constitutive Erk activation in IGF-IR overexpressing cells induced phenotypic changes similar to those observed with Akt1 downregulation. Furthermore, Erk inhibition reversed the enhancement in cell motility caused by Akt1 downregulation. These results indicate that there is a dichotomy in the activities of Akt isoforms, with Akt1 playing a negative modulatory role by suppressing Erk activation and masking induction of EMT, and Akt2 positively affecting cell proliferation and cell survival

779

**Integrin-specific Mechanisms Regulate Cell Responses to a Provisional Matrix**

H. C. Hsia, L. V. Valenick, S. A. Williams, J. E. Schwarzbauer; Molecular Biology, Princeton University, Princeton, NJ

Connections between the extracellular matrix (ECM) and intracellular compartments occur through integrins and other transmembrane receptors. To dissect the roles of cell adhesive ECM proteins in regulating cell functions, we are using a three-dimensional fibrin-fibronectin matrix, resembling the wound provisional matrix. Fibroblasts adhere and spread on this matrix using α5β1 integrin-fibronectin interactions with concomitant engagement of syndecan-4, a transmembrane proteoglycan receptor that also binds to fibronectin. This interaction promotes cell adhesion, actin stress fiber formation, and activation of focal adhesion kinase (FAK) and Rho GTPase. When the ECM protein tenascin-C is present in the matrix, syndecan-4 binding to fibronectin is reduced which prevents FAK and Rho signaling and promotes extension of filopodia, typical of a motile cell phenotype. Tenascin-C also impacts cell contractility. Fibroblasts induced to express tenascin-C using a recombinant adenovirus were deficient in the ability to contract a fibrin-fibronectin matrix but were rescued by activation of Rho with lysophosphatidic acid. Thus tenascin-C affects cell-matrix interactions and signaling via both paracrine and autocrine mechanisms. Another integrin receptor for fibronectin α4β1 does not naturally support strong cell interactions with a fibrin-fibronectin matrix. Surprisingly, however, α4β1 interactions with this matrix are dramatically enhanced, to levels equivalent to α5β1, when the fibronectin is cleaved by proteases. Fibronectin fragmentation apparently generates new sites for α4β1 binding, an effect that could play an important role in matrix remodeling in vivo. Thus, deposition of additional ECM components, expression of co-receptors for ECM, and cleavage of adhesive proteins are different ways to modulate cell responses to fibronectin matrix.

780

**The Small-G Protein Rap1 Regulates Breast Acinar Polarity in Human Epithelial Cells**

M. Itoh, C. A. Myers, M. J. Bissell; Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

Multiple cellular processes are dysregulated in tumor formation and progression. Although commonly described as a disease of unrestricted cell proliferation, a defining feature of epithelium-derived cancer is the loss of normal tissue architecture. The finely coordinated integration of a numbers of processes including cell-cell and cell-ECM interactions, polarity, survival, and proliferation are required for the maintenance of normal tissue architecture. Dysregulation of any of these processes could disrupt tissue organization and lead to tumor formation and progression. To determine the molecular mechanisms involved in breast tumor progression and reversion, we employed a three-dimensional laminin-rich extracellular matrix (3D lrECM) assay with a human breast tumor progression series, which allows us to investigate tumor progression in the context of relevant tissue architecture. We find that Ras-related small-G protein Rap1 activity plays a role in malignant phenotype of T4-2 human breast cancer epithelial cells. Rap1 activity is up-regulated in malignant T4-2 cells compared to non-malignant counterpart S1 cells in 3D lrECM culture. Additional activation of Rap1 enhances the malignancy of T4-2 cells; cell invasiveness and tumorigenesity *in vivo* is increased by introducing dominant-active Rap1. In addition, cells expressing dominant-active Rap1 escape from growth suppression and structural reorganization by treatment with AG1478 which inhibits EGFR signaling in 3D lrECM. On the other hand, reducing Rap1 activity in T4-2 cells causes reversion to a non-malignant acinar-like phenotype; structures derived from dominant-negative Rap1-expressing T4-2 cells show growth-arrested and polarized acinus-like structures in 3D lrECM similar to those derived from normal mammary epithelial cells. Both ERK and AKT pathways appear to be involved in these phenotypic alterations of T4-2 cells. These results suggest that increased Rap1 activity influences breast tumor progression through loss of organization of tissue architecture.

781

**Reconstruction of the Mammary Branching Morphogenesis Program in 3D Organotypic Cultures**

A. J. Ewald, Z. Werb; Anatomy, University of California, San Francisco, San Francisco, CA

The mammary gland forms through the invasion and branching of epithelium through a fatty stroma. We have combined in vivo genetic analysis with a 3D culture model of mammary branching morphogenesis to dissect the interactions between growth factor signaling, extracellular matrix (ECM) environment, and ECM modifying proteases during mammary development. We have reconstructed the program of mammary branching morphogenesis in these cultures from long-term time-lapse movies. In response to growth factor signals, we observe epithelial remodeling and compaction, lumen formation through cell death, ductal initiation, extension, and bifurcation. Duct initiation and extension occurs by collective sheet movement rather than migration of individual cells. The branching program is critically dependent on the 3D ECM environment. Equivalent cells in 2D do not exhibit this branching behavior and changing the ECM environment from collagen I to Matrigel dramatically changes branching behavior. The interpretation of the 3D ECM environment and growth factor signal requires protease activity, which we have demonstrated through pharmacological and genetic means. Epithelium from the same mouse, with the same ECM and media, but different protease activity, exhibits completely different branching behavior and final morphology. We next examined matrix metalloproteinase (MMP) -2 and -3 null mice. Previous work from our lab (Wiseman, JCB, 2003) demonstrated that MMP2 was required for primary duct invasion and MMP3 for side branching in vivo. We observe deficient branching in the MMP2 null cultures and normal branching in the MMP3 null cultures, arguing that the cultures model primary invasion. We are currently applying time-lapse confocal analysis and pharmacological inhibition to distinguish the relative contributions of proliferation, cell movement, and matrix degradation during epithelial invasion and bifurcation in normal and MMP deficient mammary epithelium.

782

### 3-D Mechanical Stress and Matrix-binding Chemokines and Growth Factors: a Mechanism of Autologous Gradient Formation

M. A. Swartz, J. D. Shields, C. E. Helm, M. E. Fleury; Integrative Biosciences Institute, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland

Cell migration and organization is largely driven by growth factors and cytokines whose efficacies typically depend on local gradients relative to the cell rather than absolute amounts. Such factors commonly bind to the extracellular matrix to be later released by cells via proteolytic enzymes. We consider here how gradients of matrix-liberated chemokines and growth factors may be shaped by very low levels of 3-D flows and, in doing so, uncover a surprising mechanism for autologous gradient generation that cleverly utilizes the combination of subtle mechanical forces and matrix-binding factors, both of which are common in vivo. Such slow flows, on the order of microns per second, exist between the blood and lymphatic capillaries and mediate the transport of macromolecules from the blood to the tissue. We utilize two in vitro experimental systems: cell migration of cells that home to lymphatics (e.g. dendritic cells and tumor cells), and endothelial capillary morphogenesis. In the latter, we utilize a fibrin-bound VEGF variant that is released proteolytically and show that low levels of interstitial flow can synergistically augment the effects of VEGF on the extent and organization of structure formation, and that flow specifically enhances VEGF liberation from the matrix in response to endothelial cell-derived proteases. We then use computational modeling to demonstrate how these slow flows can bias the cell-secreted protease gradients, which in turn lead to skewing of the resulting morphogen gradient and the creation of increasing (autocrine-derived) gradients. In contrast, diffusion alone can only account for decreasing, symmetric autocrine gradients. This work thus demonstrates a novel mechanism of autologous gradient generation and amplification by subtle and ubiquitous mechanical forces.

## Trafficking Proteins & Complexes (783-788)

783

### Endocytosis at the Synapse: Clathrin and Endophilin-dependent Mechanisms

H. T. McMahon; Neurobiology Division, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Clathrin-coated vesicles are found in synapses after extensive stimulation of exocytosis. By examining the networks of protein interactions and the usage of membrane bending modules (ANTH, ENTH, BAR and N-BAR) in this pathway we discovered that a parallel pathway operates for which the molecules are already discovered, but have been miss-assigned. Thus we can now show a role for endophilin in fast vesicle recycling at the synapse that is independent of AP2 adaptor/clathrin-coated vesicle formation. Use of a dominant negative endophilin inhibits fast vesicle retrieval, as measured by capacitance in goldfish bipolar terminals, but the clathrin pathway compensates with a time constant at least 10 fold slower. We can also show how other BAR domain protein are involved in other vesicle budding pathways. This has led us to try and understand what is so special about coated vesicle formation. Thus I will present a view of the hubs and nodes of the endocytic interactome, how these hubs function and how the network works to generate vesicles of uniform size and high fidelity. (For more information see www.endocytosis.org) Praefcke et al (2004) EMBO J. 23, 4371-4383. A paper on hubs in clathrin-mediated endocytosis Peters et al (2004) Science 203, 495-499. A paper on the structure and function of BAR domains Ford et al (2002) Nature 419, 361-366. A paper on the structure and function of ENTH domains

784

### Connecdenn, an AP-2-Binding Protein Linking DENN Domains to Clathrin-Mediated Trafficking in the Synapse

P. D. Allaire,[1] B. Ritter,[1] J. L. Burman,[1] S. Thomas,[1] A. Denisov,[2] K. Gehring,[2] P. S. McPherson[1]; [1]Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, PQ, Canada, [2]Biochemistry, McGill University, Montreal, PQ, Canada

Proteins of the endocytic network interact with each other and with membranes through small peptide motifs and modular protein domains. We now describe a new component of the endocytic machinery, termed connecdenn, which contains multiple motifs for binding the α-ear of the clathrin adaptor protein 2 (AP-2). Connecdenn co-immunoprecipitates and co-localizes with AP-2, and peptide competition studies coupled with nuclear magnetic resonance analysis of the α-ear in a complex with a connecdenn peptide define its interaction with the adaptor. In addition to AP-2-binding motifs, connecdenn contains multiple SH3 domain-binding motifs and interacts with SH3 domains of the synaptic proteins intersectin and endophilin. Connecdenn is highly enriched on neuronal clathrin-coated vesicles (CCVs) and is present in the pre-synaptic compartment of neurons. Overexpression of connecdenn disrupts clathrin-mediated endocytosis. At its N-terminus, connecdenn bears the uncharacterized modular DENN (differentially expressed in normal versus neoplastic tissues) domains, which are evolutionarily conserved and are found in multiple proteins with distinct functions. The DENN domains of connecdenn are responsible for stable interactions of the protein with CCVs stripped of their clathrin coats. We thus provide the first demonstration of a functional role for DENN domains and through connecdenn we link DENN domains to the endocytic machinery.

785

### An SH3 Domain in an Endocytic Protein Binds to Ubiquitin to Regulate Protein-Protein Interactions

L. Hicke, S. D. Stamenova, S. A. Francis; Biochemistry, Molecular Biology & Cell Biology, Northwestern University, Evanston, IL

Ubiquitin is a small protein that becomes covalently attached to other proteins to regulate their quantity, location or activity. In the endocytic pathway, ubiquitination targets cargo proteins for sorting into vesicles and regulates components of the endocytic machinery. To understand how ubiquitin regulates endocytosis, we identified yeast proteins required for receptor internalization that bind to monoubiquitin. Yeast epsins (Ent1 and Ent2) and the Eps15 homologue, Ede1, bind to ubiquitin through well-characterized ubiquitin-binding domains (UBDs). By contrast, yeast Sla1 (CIN85 homologue) and Rvs167 (endophilin/amphiphysin homologue) bind to ubiquitin through novel ubiquitin-binding motifs. Sla1 binds to ubiquitin through one of its three N-terminal SH3 domains. Binding of this domain, SH3-3, to ubiquitin is direct and specific. SH3-3 does not bind to canonical PXXP peptide sequences. The ubiquitin-binding site on the SH3-3 domain is composed amino acids on the hydrophobic groove that is the PXXP binding site in other SH3 domains. One important determinant of ubiquitin-binding is the presence of Phe in a position in the SH3-3 groove that is occupied by Tyr in most SH3 domains, including Sla1 SH3-1 and SH3-2. The third SH3 domain of human CIN85 has a Phe in this key position and also binds to ubiquitin. The CIN85 SH3 domain binds less well than Sla1 SH3-3, suggesting that other features of a SH3 domain are also important in determining ubiquitin affinity. Genetic evidence for functional redundancy of SH3-3 with another protein-protein interaction

domain in Sla1 implies that SH3-3-ubiquitin binding is important for regulation of endocytic network complex formation. We propose that multiple UBD-ubiquitin interactions between different proteins overlap in function with other protein-protein interactions, such as EH (Eps15 homology) domain-NPF and SH3-PXXP, to connect and regulate endocytic proteins at the plasma membrane.

786

**Biochemical and Structural Analysis of the Rab/GEF Interaction**

A. Delprato; Department of Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

Rab GTPases play a critical role in the membrane biology of eukaryotic cells by mediating processes such as vesicle budding, cargo sorting, and vesicular transport as well as the priming, docking, and fusion of vesicles with target membranes. Guanine nucleotide exchange factors (GEFs) are structurally diverse proteins that activate small GTPases by catalyzing the exchange of GDP for GTP. Despite the large number of Rab GTPases and their functional importance in membrane trafficking, remarkably little is known about the structural basis by which Rab GEFs recognize and activate Rab GTPases, or the mechanisms for allosteric regulation of Rab GEF activity. Rabex-5 catalyzes nucleotide exchange on Rab5 and cooperates with other factors to promote endosome fusion. We have previously shown that the catalytic domain of Rabex-5 also catalyzes nucleotide exchange on Rab21, which co-localizes with Rab5 on endosomes. In this study, we have solved the structure of the catalytic helical bundle -Vps9 domain of Rabex-5 in complex with nucleotide free Rab21. The crystal structure of the Rabex-5/Rab21 complex is strikingly similar to the Sec7/Arf GTPase complex and provides critical information regarding the conformational changes that accompany the activation of Rab GTPases as well as the structural basis for Rab recognition by Vps9 domains. We have also identified a potent auto-inhibitory region in Rabex-5 that accounts for the weak exchange activity of the full-length protein.

787

**The Structures of Exo70p and the Exo84p C-terminal Domains: a Common Motif and Implications for Exocyst Architecture**

G. Dong, A. H. Hutagalung, C. Fu, P. Novick, K. M. Reinisch; Cell Biology, Yale School of Medicine, New Haven, CT

The exocyst is a large tethering complex that is required for tethering vesicles at the final stages of the exocytic pathway in all eukaryotes. Here we present the structures of the Exo70p subunit and the Exo84p C-terminal domains of the exocyst complex from *S. cerevisiae*, at 2.0 Å and 2.85 Å resolution, respectively. Exo70p forms an elongated rod of 160 Å in length and 30-35 Å in width. The structure shows a novel fold that is composed of four alpha helical bundles arranged sequentially from N- to C-terminus along the length of the rod. The Exo84p C-terminus also forms a long rod (80 Å) consisting of two alpha helical bundled domains, which unexpectedly has the same fold as the Exo70p N-terminus. Our *in vitro* binding experiments demonstrate that Exo70p interacts with three other exocyst subunits, Sec6p, Sec8p, and Sec10p. Whereas the interaction with Sec6p requires only the third domain, that with Sec8p or Sec10p requires most of the sequence of Exo70p. These extended interaction surfaces for Sec8p and Sec10p on Exo70p, stretching 120-160 Å, suggest that these two proteins are also elongated. Combining knowledge of the high resolution structures of exocyst subunits, low resolution images obtained by electron microscopy, and bioinformatic analysis of all exocyst subunits, we propose that many of the exocyst subunits are comprised of helical "exoblock" modules strung together into long rod-like structures. Finally, our experiments probing the interaction between Exo70p and Rho3 GTPase narrow the Rho3p binding site to the third domain of Exo70p. This Exo70/Rho3 interaction may help to tether the entire exocyst to the plasma membrane. A conserved, positively charged surface patch at the tip of the Exo70p C-terminus may also play a role in membrane association.

788

**Structural Mechanism for Sterol Sensing and Transport by OSBP-related Proteins**

Y. Im,[1] S. Raychaudhuri,[2] W. A. Prinz,[2] J. Hurley[3]; [1]Laboratory of Molecular Biology, NIDDK/NIH, Bethesda, MD, [2]Laboratory of Cell Biochemistry and Biology, NIDDK/NIH, Bethesda, MD, [3]Structural Biology and Cell Signaling, NIDDK/NIH, Bethesda, MD

The oxysterol binding protein (OSBP)-related proteins (ORPs) are conserved from yeast to man and are implicated in regulation of sterol pathways and in signal transduction. The structure of the full-length yeast ORP Osh4 was determined at 1.5-1.9 Å resolution in complexes with ergosterol, cholesterol, and 7-, 20-, and 25-hydroxycholesterol. A single sterol molecule binds in a hydrophobic tunnel in a manner consistent with a transport function for ORPs. The entrance is blocked by a flexible N-terminal lid and surrounded by functionally critical basic residues. The structure of the open state of a lid-truncated form of Osh4 was determined at 2.5 Å resolution. Structural analysis and limited proteolysis show that sterol binding closes the lid and stabilizes a conformation favoring transport across aqueous barriers and transmitting signals. The unliganded structure exposes potential phospholipid-binding sites that are positioned for membrane docking and sterol exchange. Based on these observations we propose a model in which sterol and membrane binding promote reciprocal conformational changes that facilitate a sterol transfer and signaling cycle.

## Signal Transduction II (789-818)

789

**Pathway and Expression Level-Dependent Effects of Oncogenic N-Ras: $p27^{Kip1}$ Mislocalization by the Ral-GEF Pathway and Erk-Mediated Interference with TGF-β Signaling**

Y. I. Henis,[1] S. Kfir,[1] M. Ehrlich,[1] A. Goldshmid,[1] X. Liu,[2] Y. Kloog[1]; [1]Neurobiochemistry, Tel Aviv University, Tel Aviv, Israel, [2]Chemistry and Biochemistry, University of Colorado-Boulder, Boulder, CO

Overactivation of Ras pathways contributes to oncogenesis and metastasis of epithelial cells in several ways, including interference with cell cycle regulation *via* the CDK inhibitor $p27^{Kip1}$ (p27) and disruption of transforming growth factor-β (TGF-β) anti-proliferative activity. Here we show that at high expression levels, constitutively active N-Ras induces cytoplasmic mislocalization of murine and human p27 *via* the Ral-GEF pathway and disrupts TGF-β-mediated Smad nuclear translocation by activation of the Mek/Erk pathway. While human p27 could also be mislocalized *via* the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, only Ral-GEF activation was effective for murine p27, which lacks the Thr157 Akt phosphorylation site of human p27. This establishes a novel role for the Ral-GEF pathway in regulating p27 localization. Interference with either Smad translocation or p27 nuclear localization was sufficient to disrupt TGF-β growth inhibition. Moreover, expression of activated N-Ras or specific effector loop mutants at lower levels using retroviral vectors induced p27 mislocalization but did not inhibit Smad2/3 translocation,

indicating that the effects on p27 localization occur at lower levels of activated Ras. These findings have important implications for the contribution of activated Ras to oncogenesis, and for the conversion of TGF-β from an inhibitory to a metastatic factor in some epithelial tumors.

790

**Constitutive Activation of MEK/ERK MAP Kinase Pathway Induces Expression and Activity of MMP2, MMP9 and SerpinE2 : Roles in Intestinal Tumorigenesis**

V. Durand, M. Boucher, J. Laine, N. Rivard; Anatomy and Cell Biology, University of Sherbrooke, Sherbrooke, PQ, Canada

Strong evidences exist for the critical involvement of Ras/Raf/MEK/ERK cascade in the regulation of intestinal epithelial cell proliferation. K-*ras*$^{V12G}$ is the most frequently mutated oncogene in colorectal cancer and targeted expression of K-ras$^{V12G}$ in the intestinal epithelium causes activation of the MEK/ERK pathway and tumorigenesis in mice. Moreover, blockade of the MEK/ERK pathway suppresses growth of colon tumors in vivo and overexpression of an active mutant of MEK1 induces transformation of cultured intestinal epithelial cells. However, the mechanisms by which the MEK/ERK cascade induces intestinal epithelial cell tumorigenesis remain to be clarified. Methods. Retrovirus encoding the HA-tagged MEK1 wild type (wtMEK) or a constitutive active mutant of MEK1 (MEK1-S218D/S222D, caMEK) were used to infect normal intestinal epithelial crypt cells IEC-6. Modifications of gene expression were determined with Affymetrix rat microarrays. Expression of specific genes was verified by RT-PCR and western blot. By zymography, we determined matrix metalloprotease (MMP) activities. Results. 1-Microarray data showed that constitutive activation of MEK/ERK cascade induces genes involved in cell adhesion and migration, including serpinE2, tissue plasminogen activator, syndecan1, integrinα6, and represses TIMP2/3 genes and genes involved in cell-cell interaction. 2- RT-PCR and western blot analysis confirm the induction of serpinE2, MMP2 and MMP9 in caMEK expressing cells and zymography studies confirm that elevated MMP2 and MMP9 activities are found in their media. 3-Treatment of caMEK expressing cells with UO126 (inhibitor of MEK1/2; 10 uM) or with MMP2/9 inhibitor IV (20 uM) markedly reduces cell proliferation, foci formation and migration (Boyden chamber) and abolishes expression/activity of serpinE2 and MMPs. Conclusion. Constitutive activation of MEK/ERK cascade regulates the expression and activity of extracellular proteases which may confer growth advantage and invasive capacity to intestinal epithelial cells.

791

**Stat3 Regulation by the Caveolae Protein, Cav-1, in Breast Cancer**

K. L. Albert,[1] R. Arulanandam,[1] A. Vultur,[1] L. Raptis,[1] R. Jove,[2] J. Turkson[2]; [1]Department of Pathology, Queen's University, Kingston, ON, Canada, [2]Moffitt Cancer Center, Tampa, FL

Caveolae are cholesterol-rich, flask-shaped invaginations of the plasma membrane with many roles in the cell including signal transduction. Stat3 is a cytoplasmic signal transducer which, upon stimulation of a large number of receptors, is activated by phosphorylation, then migrates to the nucleus to initiate transcription of genes involved in cell division and survival. Despite extensive evidence on the role of caveolae in signal transduction, their effect upon Stat3 is still obscure. We previously demonstrated that cell-to-cell adhesion, as occurs in confluent cultures, can cause a dramatic increase in Stat3 phosphorylation and activity in cultured normal human breast and carcinoma cells. Examination of the nature of the kinases involved revealed that pharmacological inhibition or genetic ablation of a number of kinases frequently found to be activated in breast cancer such as Src, Fyn, Yes, EGFR, Fer and IGF1-R did ***not*** prevent this Stat3 activation, indicating that these kinases ***individually*** do not play a significant role. Given that many kinase inhibitors were found to be ineffective in clinical trials, we attempted to examine the mechanism of this cell-cell adhesion-mediated, Stat3 activation. Our results indicated that cav-1 transcriptional downregulation results in a dramatic activation of Stat3, which points to cav-1 as an inhibitor of Stat3 activity. Moreover, our data revealed that cell-to-cell adhesion causes a dramatic drop in cav-1 expression, which is followed by a sharp Stat3 activity increase. Therefore, it appears that the activation of a multitude of kinases through cav-1 downregulation at post-confluence might be responsible for the Stat3 activity increase, as well as the absence of Stat3 inhibition through inactivation of specific kinases individually. This work may expose the caveolae as an essential target for the treatment of tumors resistant to multiple kinase inhibitors.

792

**Mechanisms Regulating Oncogenic Signalling by Sphingosine Kinase**

S. M. Pitson; Human Immunology, IMVS, Adelaide, Australia

Sphingosine kinase 1 (SK1) catalyzes the formation of the bioactive lipid sphingosine 1-phosphate, and has been implicated in a number of biological processes in mammalian cells, including enhanced proliferation, inhibition of apoptosis, and oncogenesis. SK1 possesses high instrinsic catalytic activity which can be further increased by a diverse array of cellular agonists. We have begun to understand the molecular mechanisms regulating oncogenic signalling by SK1 by recently demonstrating that; (i) this activation occurs as a direct consequence of ERK1/2-mediated phosphorylation at Ser225, which not only increases catalytic activity, but is also necessary for agonist-induced translocation of SK1 from the cytoplasm to the plasma membrane; (ii) the oncogenic effects of overexpressed SK1 are blocked by mutation of the phosphorylation site despite the phosphorylation-deficient form of the enzyme retaining full instrinsic catalytic activity, and; (iii) SK1 translocation is the key effect of phosphorylation in oncogenic signalling by this enzyme through constitutive localization of the phosphorylation-deficient form of SK1 to the plasma membrane. We now show that calmodulin may play an essential role in regulating the important process of SK1 localisation since disruption of the CaM-binding site of this enzyme ablates its agonist-induced translocation to the plasma membrane.

793

**Role of A-Kinase Anchoring Proteins in cAMP-mediated Schwann Cell Proliferation**

A. L. Asirvatham,[1] C. M. Schworer,[2] D. Heitzman,[2] D. J. Carey[2]; [1]Biology, College Misericordia, Dallas, PA, [2]Weis Center for Research, Geisinger Clinic, Danville, PA

Proliferation of Schwann cells during peripheral nerve development is stimulated by the heregulin/neuregulin family of growth factors expressed by neurons. However, for neonatal rat Schwann cells growing in culture, heregulins produce only a weak mitogenic response. Supplementing heregulin with forskolin, an agent that elevates cyclic AMP levels but has no mitogenic response itself, produces a dramatic increase in the proliferation of cultured Schwann cells. The mechanisms underlying this synergistic effect are not understood, nor is it established if cyclic AMP

elevation is required for Schwann cell proliferation *in vivo*. Characterizing the A-kinase anchoring proteins (AKAPs) in Schwann cells might help identify substrates tethered to and phosphorylated by PKA. Using an RII overlay assay that detects AKAPs, we identified AKAP150 in Schwann cells. Western blot analysis confirmed the presence of AKAP150 and revealed that additional AKAPs, specifically, AKAP95, Yotiao and Ezrin, were also present. Immunoblot analysis indicated that the steady-state expression of AKAP150, AKAP95, and Ezrin was not regulated by treatment with heregulin or forskolin. We also utilized RNA interference technology to specifically knockdown AKAP150 and AKAP95. Transfection with sequence-specific AKAP siRNAs revealed a 97% and 80% reduction for AKAP95 and AKAP150, respectively, after 24 hours. Morphological characterization of Schwann cell AKAPs indicated the presence of nuclear (AKAP95) and membrane-associated (AKAP150) A-kinase anchoring proteins. Preliminary data suggest a role for AKAP95 and AKAP150 in the synergistic response of Schwann cells to treatment with heregulin and forskolin.

794

**Inhibition of Cell Proliferation by Chk Tyrosine Kinase that Localizes to the Nucleus**

Y. Nakayama, A. Igarashi, A. Kawana, N. Yamaguchi; Dept. of Mol. Cell Biol., Grad. Sch. of Pharm. Sci., Chiba University, Chiba, Japan

Csk-family tyrosine kinases, Csk and Chk, are known to suppress the activity of Src-family kinases by tyrosine phosphorylation at the C-terminal tail. Recently, we found that Chk is distributed to the nucleus in addition to the cytoplasm whereas the distribution of Csk is restricted to the cytoplasm. Expression of Chk induced inhibition of cell proliferation. In this study, to investigate the role of Chk in cell proliferation, we analyzed Chk-induced tyrosine phosphorylation in the nucleus and nuclear multi-lobulation. We observed that expression of Chk induced tyrosine phosphorylation in the nucleus in HeLa cells. The experiments with the N-terminal domain-lacking ChkΔN mutant showed that the presence of the N-terminal domain led to a fourfold increase in the cell population exhibiting tyrosine phosphorylation in the nucleus through association of Chk with the nucleus. The tyrosine phosphorylation was induced in a manner that was dependent on the kinase activity of Chk. In situ subnuclear fractionations revealed that Chk induced tyrosine phosphorylation of DNA-associated and nuclear matrix proteins. We also found that the kinase-active but not -inactive Chk mutant inhibited cell proliferation, suggesting that tyrosine phosphorylation induced by Chk plays an important role in Chk-induced inhibition of cell proliferation. Intriguingly, nuclear localizing Chk mutants prolonged S phase of the cell cycle accompanying dynamic nuclear multi-lobulation in a kinase acitvity-independent manner. Cell sorting analysis revealed that nuclear multi-lobulated cells were enriched in late S phase. Multi-lobulated nuclei were surrounded with aberrantly distributed lamin B1, and the multi-lobulation was dependent on microtubules. In conclusion, our findings provide evidence that Chk-induced inhibition of cell proliferation involves the kinase activity-dependent and -independent mechanisms in the nucleus.

795

**Novel Pathway of Rac1, Stat3 and p53 Interaction**

A. Anagnostopoulou,[1] R. Arulanandam,[1] A. Vultur,[1] J. Turkson,[2] R. Jove,[2] L. Raptis[1]; [1]Pathology, Queen's University, Kingston, ON, Canada, [2]Moffitt Cancer Center, Tampa, FL

Stat3 is activated by a number of membrane tyrosine kinases known to be activated in breast cancer. We recently demonstrated that cell-cell adhesion, such as brought about through confluence, can lead to a dramatic increase in Stat3 activity, despite the pronounced growth inhibition. This activation was independent from a number of kinases, including the Src family, EGFR, IGF1-R and Fer, which are often activated in breast cancer. It is also established that cell-cell adhesion may result in the rapid activation of the Rac1 GTPase and that v-Rac can activate Stat3. Our results showed that density did, in fact, stimulate Rac1 activity. However, we also discovered a dramatic increase in total Rac1 protein levels. This increase preceded the Stat3 activity increase by approximately 24 hours, indicating that the density-mediated Rac1 activation could explain the dramatic upregulation in Stat3 activity. Loss of function of the p53 transcription factor is critical for the development of malignancies. However, most natural p53 mutations occur at a late stage in tumor development, and the majority of clinically detectable breast cancers lack p53 mutations. Nevertheless, reduced p53 levels are observed instead. Recent results revealed that Stat3 interacts with the p53 promoter and downregulates p53 transcription. We now demonstrate that the Stat3 increase seen at post-confluence is followed by a dramatic decrease in p53 levels. Furthermore, interrupting Stat3 signaling in densely growing cells through the use of pharmacological inhibitors or genetic ablation up-regulates p53 expression, leading to p53-mediated tumor cell apoptosis. Our findings thus identify Stat3 as a molecular target for reactivating p53 in malignancies such as the majority of breast cancers that still retain an intact p53 gene and could have important implications for the treatment of cancers where inhibitors of tyrosine kinases would be ineffective.

796

**Phosphorylation of Focal Adhesion Kinase At Tyr397, Tyr861, and Tyr925 is involved in Transendothelial Migration of AU-565 Breast Cancer Cells**

S. Earley, G. E. Plopper; Biology, Rensselaer Polytechnic Institute, Troy, NY

The final step of the metastatic cascade involves extravasation of cancer cells through the endothelial lining of blood vessels. AU-565 metastatic breast cancer cells migrate through a bovine lung microvessel endothelial cell (BLMVEC) monolayer, as shown using filter-based migration assays as well as electric cell-substrate impedance sensing (ECIS). Focal adhesion kinase (FAK) is involved in this process, as transient transfection of AU-565 breast cancer cells with siRNA that targets FAK mRNA results in approximately 50% knockdown FAK mRNA expression and diminished transendothelial migration of AU-565 breast cancer cells. Expression of the dominant negative FAK inhibitor FRNK or FAK point mutants (Y397F, Y861F, and Y925F) also results in decreased AU-565 transendothelial migration, whereas overexpression of wildtype FAK or transfection with an empty vector does not. These results demonstrate the importance of FAK signaling in migration of cancer cells across endothelial cell boundaries.

797

**Activated AKT Regulates Nf-κB Activation, P53 Inhibition and Cell Survival in HTLV-1-transformed Cells**

S. Jeong, C. A. Pise-Masison, M. F. Radonovich, H. Park, J. N. Brady; National Cancer Institute, National Institutes of Health, Bethesda, MD

AKT activation enhances resistance to apoptosis and induces cell survival signaling through multiple downstream pathways. Here, we present

evidence that AKT is activated in HTLV-1-transformed cells and that Tax activation of AKT is linked to NF-κB activation, p53 inhibition and cell survival. Overexpression of AKT wild type (WT), but not a kinase dead (KD) mutant resulted in increased Tax-mediated NF-κB activation. Blocking AKT with the PI3K/AKT inhibitor LY294002 or AKT SiRNA prevented NF-κB activation and inhibition of p53. Treatment of C81 cells with LY294002 resulted in an increase in the p53-responsive gene MDM2, further suggesting a role for AKT in the Tax-mediated regulation of p53 transcriptional activity. We show that LY294002 treatment of C81 cells abrogates *in vitro* IKKβ phosphorylation of p65 and causes a reduction of p65 Ser-536 phosphorylation *in vivo*, steps critical to p53 inhibition. Interestingly, inhibition of AKT function did not affect IKKβ phosphorylation of IκBα *in vitro* suggesting selective activity of AKT on the IKKβ complex. Finally, we present evidence that AKT plays a pro-survival function in HTLV-1-transformed cells, which is linked to expression of the Bcl-xL gene. We suggest that AKT plays a role in activation of pro-survival pathways in HTLV-1-transformed cells, through NF-κB activation and inhibition of p53 transcription activity.

798

**Mechanical Regulation of Gene Expression Through the Cysteine-rich Protein 61 (Cyr61) in Smooth Muscle Cells**

D. Zhou,[1] D. Herrick,[2] J. Rosenbloom,[3] B. Chaqour[4]; [1]Dept of Anatomy and Cell Biology, University of Pennsylvania, Philadelphia, PA, [2]Thomas Jefferson University, Philadelphia, PA, [3]University of Pennsylvania, Philadelphia, PA, [4]Dept of Anatomy and Cell Biology, State University of New York (SUNY) Downstate Medical Center, Brooklyn, NY

Application of cyclic strain to in vitro cultured smooth muscle (SM) cells results in profound alterations of the histo-morphometry, phenotype and function of the cells. The onset of this process is characterized by the activation of a cascade of signaling events coupled to progressive and perhaps, interdependent changes of gene expression. In particular, externally applied cyclic stretch to cultured bladder SM cells results in the transient expression of the Cyr61 gene that encodes a cysteine-rich extracelluar matrix-associated heparin-binding protein originally described as a proangiogenic factor capable of altering the gene programs for angiogenesis, adhesion and extracellular matrix synthesis. In this study, we investigated the effects of mechanical stretch-induced Cyr61 on the expression of potential mechano-sensitive Cyr61 target genes and the signaling pathways involved. We showed that suppression of Cyr61 expression with an adenoviral vector encoding an anti-sense oligonucleotide reduced mechanical strain-induced VEGF, $\alpha_v$ integrin and SM α-actin gene expression but had no effect on myosin heavy chain (MHC) isoforms, SM-1 and SM-2. Signaling pathways involving RhoA GTPase, phosphatidyl inositol 3-kinase and cytoskeletal actin dynamics altered stretch-induced Cyr61 and Cyr61 target genes. Reciprocally, adenovirus-mediated overexpression of Cyr61 in cells cultured under static conditions increased the expression of VEGF, $\alpha_v$ integrin and SM α-actin, as well as that of SM-1 and SM-2 isoforms suggesting that the effects of a sustained expression of Cyr61 extend to SM specific contractile function. These effects were dependent on the integrity of the actin cytoskeleton. Together, these results indicate that Cyr61 is an important determinant of the genetic reprogramming that occurs in mechanically challenged cells.

799

**SUMO-1 Modification Influences SnoN to Regulate Myogenesis**

X. Feng,[1,2] K. Wrighton,[2] M. Liang,[1] B. Bryan,[3] K. Luo,[4] M. Liu,[3] X. Lin[2]; [1]Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX, [2]Surgery, Baylor College of Medicine, Houston, TX, [3]Alkek Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX, [4]Department of Molecular Cell Biology, University of California, Berkeley, CA

SnoN and Ski control transformation and myogenic differentiation. SnoN/Ski induce transformation by repressing TGF-β signaling, but how they function in myogenesis is largely unknown. Herein we identify SnoN as a target for SUMO-1 modification. Sumoylation occurs primarily at lysine 50 (K50). PIAS family E3 ligases physically interact with SnoN to stimulate its sumoylation. SnoN sumoylation does not alter its stability or its ability to repress TGF-β signaling. However, mutation of SnoN at the K50 sumoylation site enhances its myogenic potential, as ascertained by its increased ability to activate muscle-specific gene expression and enhance myotube formation. Our study identifies a novel role for sumoylation in the regulation of differentiation.

800

**Sodium-Hydrogen Exchanger Isoform 1 (NHE1) Activity Modulation via Physical Interaction by Daxx**

H. Kim,[1] Y. Jung,[1] J. O. Kim,[2] M. G. Lee,[2] E. Kim[1]; [1]School of Bioscience and Biotechnology, Chungnam National University, Daejon, Republic of Korea, [2]Department of Pharmacology and Brain 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea

NHE1 is a $Na^+/H^+$ antiport protein that regulates the intracellular pH and is activated upon ischemic injury. This study reports that Daxx interacts with NHE1 and modulates its channel activity upon ischemic injury. Both N and C termini of Daxx were mapped as NHE1 binding regions and the cytosolic domain of NHE1 was responsible for the interaction with Daxx. The effect of Daxx-NHE1 interaction was assessed upon ischemic injury in H9c2 cells and in PS120-NHE1, a PS120 cell which stably overexpresses NHE1. Daxx translocates from the nucleus to the cytosol upon ischemia in H9c2 cells. We used Daxx(W621A) mutant which is confined to the cytosol because cytoplasmic Daxx could be able to contact NHE1. Overexpression of Daxx(W621A) increased the NHE1 channel activity in PS120-NHE1 cells. However, Daxx(S667A) which is confined to the nucleus failed to do so. We also have shown that Daxx(W621A) enhances ischemic cell death whereas Daxx(S667A) blocks the ischemic cell death. Thus, our data suggest that Daxx can modulate NHE1 channel activity via nuclear export. [Supported by the grant (CBM2-B211-001-1-0-0) from the Center for Biological Modulators of the 21st Century Frontier R&D Program, MOST, Korea]

801

**Cell Cycle-regulated Changes in S6K2 Localization and Activity**

M. McDowell,[1] R. Rossi,[2] D. Boyer,[1] J. Pester,[1] A. Montecucco,[2] K. Lee-Fruman[1]; [1]Biological Sciences, California State University Long Beach, Long Beach, CA, [2]Istituto di Genetica Molecolare (IGM) - CNR, Pavia, Italy

Ribosomal S6 kinase 2 (S6K2) is a serine/threonine kinase that was identified based on its homology to S6 kinase 1 (S6K1). The cellular function of S6K2 is yet to be determined, and the localization of endogenous S6K2 protein has not been studied. To better understand the role of S6K2, we carried out detailed temporal and spatial subcellular localization and activity studies via conventional and confocal immunofluorescence as well as with immune complex kinase assay. In HeLa cells S6K2 is found in a punctate pattern both in the cytoplasm and in the nucleus during G1 phase

whereas it is confined to the cell nucleus in S and G2 phases. Immunocolocalization experiments show that a fraction of S6K2 colocalizes with γ-tubulin at the centrosome in G1 phase and at the mitotic spindle poles. Translocation of S6K2 to centrosome during G0-G1 transition is stimulated by various mitogens and is regulated by mammalian target of rapamycin (mTOR). Although cytoplasmic translocation is seen immediately after mitogen is added to cells, S6K2 activity was greater in late G1 and S phases than in early G1 phase, indicating that although S6K2 is confined to the nucleus in S phase it is nevertheless active. We also show that a fraction of S6K2 nuclear foci colocalizes with coilin and fibrillarin at Cajal bodies but not at nucleoli. Our data indicate that subcellular localization of S6K2 is complex and dynamically controlled during the cell cycle. This is the first report of S6K2 as a kinase that associates with the centrosome and Cajal body.

802

**The Multisubstrate Adaptor Protein Gab1 is Involved in VEGF-Induced Signaling and is Required for Endothelial Cell Migration**

M. Laramée,[1] C. Chabot,[1] M. Cloutier,[1] M. Holgado-Madruga,[2] A. J. Wong,[2] I. Royal[1]; [1]Centre de recherche du CHUM, University of Montreal, Montreal, PQ, Canada, [2]Departments of Microbiology and Immunology and Pharmacology, Kimmel Cancer Institute, Philadelphia, PA

VEGF is a multifunctional cytokine involved in the promotion of endothelial cell proliferation, survival, migration, vessel permeability and morphogenesis. The VEGFR2 receptor tyrosine kinase is the main receptor responsible for conveying the biological effects of VEGF in endothelial cells. Upon stimulation, VEGFR2 associates with, and/or leads to the activation of numerous targets including phospholipase C gamma (PLCγ), Phosphatidylinositol 3-kinase (PI3K), Akt, Src and ERK/MAPK which have been involved in the promotion of endothelial cell migration. However, the VEGFR2 proximal signaling events leading to the activation of these pathways in response to VEGF stimulation remain ill-defined. We have identified the Gab1 adaptor protein as a novel VEGFR2 substrate. Gab1 encompasses a PH domain and contains a large number of tyrosine residues and proline-rich regions allowing its interaction with proteins harboring SH2 and SH3 domains. In bovine aortic endothelial cells (BAECs), Gab1 associates in a constitutive manner with Grb2 and PI3K, and in a VEGF-dependent fashion with SHP2, Shc and PLCγ. Gab1 also associates with VEGFR2 in a basal manner, and this association is increased upon VEGF stimulation. Transfection of human microvascular endothelial cells (HMVECs) with Gab1 siRNAs leads to the impaired activation of Src, PLCγ and ERK, but not that of Akt, and to the block of VEGF-induced cell migration. We further show that this is not due to the reduced adhesion, survival or proliferation of the Gab1-depleted cells. Consistent with these data, co-expression of VEGFR2 with a Gab1 dominant negative mutant unable to bind SHP2 (Gab1ΔSHP2) in HEK 293 cells mimics the results obtained with Gab1 siRNAs. Our data thus identify Gab1 and associated SHP2 as novel regulatory components of VEGF-induced signaling pathways and of endothelial cell migration.

803

**Inhibition of Osteoclast Differentiation by Sauchinone, a Lignan from *Saururus chinensis***

K. Han, E. Chang, H. Kim; Seoul National Univesity, Seoul, Republic of Korea

Osteoclasts, multinuclear cells specialized for bone resorption, differentiated from the monocyte/macrophage lineage of hematopoietic cells. Intervention in osteoclast differentiation is considered an effective therapeutic approach to the treatment of bone diseases involving osteoclasts. In this study, we investigated the inhibition effect of sauchinone, a lignan from *Saururus chinensis*, on receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclastogenesis. Addition of sauchinone to the osteoclast precursor culture caused a significant decrease in the level of calcitonin receptor, cathepsin K and TRAP mRNA, which are normally upregulated during the osteoclast differentiation dependent on RANKL. RANKL activated the ERK, p38, and NF-κB signal transduction pathways in osteoclast precursor cells, and sauchinone suppressed this activation. Sauchinone also suppressed expression of NFATc1 of differentiated osteoclasts, a master switch in osteoclastogenesis. Taken together, the data demonstrate that sauchinone inhibited RANKL-induced osteoclastogenesis and our findings may be critical for developing a strategy to ameliorate bone resorption diseases.

804

**P2 Purinergic Receptors Signal to STAT3 in Astrocytes**

K. B. Washburn,[1] J. T. Neary[2]; [1]Pathology, University of Miami, Miller School of Medicine, Miami, FL, [2]Pathology, Biochemistry & Molecular Biology, University of Miami, Miller School of Medicine, Miami, FL

Damage to the central nervous system can evoke a reactive astrogliotic response whereby normally quiescent astrocytes become proliferative. Extracellular ATP is released upon tissue damage and induces DNA synthesis in astrocytes by activating P2 purinergic receptors; however the signaling pathways associated with this response remain to be fully elucidated. Signal Transducer of Activated Transcription 3 (STAT3) has been implicated in reactive astrogliosis and plays an important role in cell cycle regulation by promoting expression of cell cycle and survival genes. Therefore, we investigated whether extracellular ATP and purinergic receptors regulate STAT3 signaling. Using immunoblot analysis, we find that addition of ATP to primary cultures of rat cortical astrocytes increases Tyr-705 phosphorylation of STAT3 and decreases Try-705 phosphorylation of STAT3b (the dominant negative splice variant) in a time-sensitive and concentration-dependent manner. ATP-stimulated Tyr-705 phosphorylation of STAT3 is mediated through P2 receptor activation since suramin, an antagonist of P2 receptors, diminishes this response, whereas 8-(para-sulfo-phenyl)-theophylline (8PSTP), an antagonist of P1 receptors, does not. The P2 class of receptors consists of both G-protein coupled receptors (P2Y) and ligand gated ion channels (P2X). Agonists for both receptor classes stimulate Tyr-705 STAT3 phosphorylation, but only activation of P2Y receptors significantly stimulates Ser-727 phosphorylation of STAT3. Because initial cytoplasmic phosphorylation of tyrosine (Tyr-705)/STAT3 enables dimerization, nuclear translocation, and DNA binding, whereas subsequent phosphorylation of serine (Ser-727) promotes DNA transcriptional activity by STAT3, this implicates P2Y, but not P2X, receptors in DNA transcription of cell cycle regulatory genes which promote DNA synthesis. Our findings indicate that P2 receptors stimulate STAT3 activation and suggest that P2 receptor/STAT3 signaling may play an important role in reactive astrogliosis. This work was supported by the Department of Veterans affairs and the National Institutes of Health.

805

**JNK-interacting Protein-3 (JIP-3) and Kinesin-1 Control a Signaling Pathway that Leads to Amyloid-*β* Precursor Protein (APP) Phosphorylation and Transport, and to Neuronal Differentiation**

Z. Muresan, V. Muresan; Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

Abnormal phosphorylation of APP is a pathologic feature of Alzheimer's disease. To begin to understand the mechanism of APP phosphorylation, we studied this process in differentiating neurons under normal, physiological conditions. We find that JNK is required for APP phosphorylation at Thr668, leading to localized accumulation of phosphorylated APP (pAPP) in neurites. We show that JIP-3, a JNK scaffolding protein that does not bind APP, selectively increases APP phosphorylation, accumulation of pAPP into processes, and stimulates process extension in both neurons and COS-1 cells. Down-regulation of JIP-3 by siRNA impairs neurite extension and reduces the amount of localized pAPP. The signaling pathway sustained by JIP-3, leading to APP phosphorylation, is qualitatively different from stress-activated JNK signaling pathways. Thus, whereas stress-activated JNK generates pAPP only in the cell body, concomitant expression of JIP-3 restores pAPP accumulation into neurites. Moreover, the JIP-3 pathway activates JNK locally, and does not trigger translocation of pJNK into the nucleus or phosphorylation of nuclear targets such as c-Jun. JIP-3 facilitated APP phosphorylation is upregulated by inhibition of Cdk5, and downregulated by inhibition of GSK 3β. Mechanistically, we found that kinesin-1 directly participates in APP phosphorylation, by bridging the interaction of JIP-3 (which is a kinesin-1 binding protein) with APP. Thus, APP phosphorylation, transport of the generated pAPP into neurites, and neurite extension are interdependent processes controlled by JIP-3/JNK, in a pathway that is modulated by Cdk5 and GSK 3β, and regulated by kinesin-1. We propose that the light chains of kinesin-1 may generally function as scaffolds for assembling signaling complexes.

806

**A Novel Role of P120 Catenin in Regulating Epithelial Cell Growth**

M. Yanagisawa, L. Marlow, J. A. Copland, P. Z. Anastasiadis; Cancer Biology, Mayo Clinic, Jacksonville, FL

E-cadherin is widely considered to be an invasion suppressor. It is also thought to regulate cell proliferation, and is likely important for contact-mediated suppression of cell growth. β-catenin, which binds the C-terminal portion of the cadherin cytoplasmic tail, may mediate these effects as it transactivates the expression of hyperproliferative genes when associated with LEF/TCF transcription factors in the nucleus. The role of p120 catenin on cell proliferation is unclear. Here, we show that p120 depletion affects the growth of E-cadherin-deficient, V12-Ras expressing MDA-MB-231 cells *in vitro*, and *in vivo*. SiRNA-mediated p120 depletion blocked the growth of MDA-MB-231 cells in soft agar and in nude mice, while re-expression of p120 isoforms 1 or 4 rescued the transformed phenotype and tumor growth. Interestingly, colonies grew as tight aggregates in p120 4A re-expressing cells, whereas they were dispersed in control MDA-MB-231 cells, or p120-depleted cells re-expressing p120 isoform 1. Furthermore, activation of Rac in p120-depleted cells rescued cell growth both in soft agar and in nude mice, consistent with a previously reported role of Rac in Ras transformation. Rac activity was blocked in p120-depleted cells and induced by either p120 isoform 1 or 4. Finally, the role of p120 depletion on MAPK and PI3K signaling was also tested. The data strongly suggest a role for endogenous p120 in Ras-mediated epithelial cell transformation and tumor growth.

807

**Role of $\alpha_v\beta_3$ Integrin in Sphingosine-1-phosphate Receptor Regulated Pro-angiogenic Activity**

L. Wang,[1] J. Lee,[2] H. Sarojini,[2] Q. Zeng,[2] E. Wang,[2] J. Cai,[3] C. Lin,[4] M. Lee[1]; [1]Microbiology and Immunology, University of Louisville Health Science Center, Louisville, KY, [2]Gheens Center on Aging, University of Louisville Health Science Center, Louisville, KY, [3]Pharmacology and Toxicology, University of Louisville Health Science Center, Louisville, KY, [4]Oncology, Georgetown University Medical Center, Washington, DC

Sphingosine-1-phosphate and integrin $\alpha_v\beta_3$ mediated signaling cascades are critical in angiogenic responses. In this report, we show integrin $\alpha_v\beta_3$ and S1P1 receptor form a functional complex in endothelial cells. The interaction between $\alpha_v\beta_3$ integrin and S1P1 receptor is specific because $\alpha_v\beta_3$ and S1P1 association is significant reduced in Lentiviral mediated S1P1 receptor silenced endothelial cells and integrin $\beta_1$ polypeptide does not interact with S1P1 receptor. Also, $\alpha_v\beta_3$ and S1P1 receptor interaction is enhanced upon sphingosine-1-phosphate stimulation and is dependent on Gi heterotrimeric G protein. In addition, sphingosine-1-phosphate treatment induces the FAK and α-actinin associating with $\beta_3$, which is dependent on $G_i$ heterotrimeric G protein and S1P1 receptor. Moreover, sphingosine-1-phosphate induced endothelial chemotaxis and morphogenesis is abrogated by the $\alpha_v\beta_3$ blocking antibody. These data together indicate that $\alpha_v\beta_3$ is a down-stream target of S1P signaling and the activation of $\alpha_v\beta_3$ integrin is critical in S1P-regulated angiogenic responses.

808

**Maintenance of the Proliferative State in Intestinal Epithelial Cells by Notch1 Signaling**

S. Guilmeau, M. Flandez, L. H. Augenlicht; Oncology, Montefiore Medical Center, New York, NY

**Objective:** To investigate whether Notch signaling is involved in the proliferation and lineage determination of intestinal epithelial cells. **Results:** *In vivo*, Notch intracellular domain 1 (NICD1), was immunolocalized to the nucleus of proliferating crypt cells in mice. Fractionation of the epithelial cells along the crypt-villus axis through the Weiser method also indicated that NICD1 was expressed in the crypt fraction of the duodenum, jenunum and ileum, with low to undetectable levels of NICD1 observed in cells from the villi. *In vitro*, we used two different cell lines that undergo spontaneous differentiation into goblet cells and enterocytes: the 16E clonal derivative of the HT29 cell line and the Caco-2 cell line, respectively. In both cell lines, NICD1 expression, as well as the Notch ligand Jagged1, were down-regulated during differentiation of the cells along either lineage. However, quantitative PCR indicated that the expression ratio of HES1, a key target gene of Notch, standardized to GAPDH, was upregulated by to 2 to 3 fold in fully differentiated cells compared to undifferentiated cells. Moreover, an MTT assay showed that a 48 hour treatment of cells with L,685-458, a gamma-secretase inhibitor (GSI), induced a dose dependent inhibition of 16E and Caco-2 cells proliferation, reaching 60 and 50% respectively, at 10uM of GSI. This growth inhibition was accompanied by a G1 arrest and an increase of cell number in the S phase. Data also suggest that RNA interference of Notch1 expression inhibited proliferation in the Caco-2 cell line. **Conclusion:** Since Notch1 signaling is activated both *in vitro* and *in vivo* in proliferating but not in differentiating cells, and its down regulation reduced proliferation *in vitro*, this pathway might be implicated in the maintenance of intestinal progenitor cells through a HES1 independent pathway.

809

**Divergence of Hedgehog Signaling between Drosophila and Mammals**

M. T. Varjosalo, S. Li, J. A. Taipale; Biomedicum, Molecular/Cancer Biology Program, University of Helsinki, Helsinki, Finland

The Hedgehog (Hh) signaling pathway has conserved roles in development of species ranging from Drosophila to Humans. Responses to Hh are mediated by the transcription factor Cubitus interruptus (Ci; GLIs 1-3 in mammals), and constitutive activation of Hh target gene expression has been linked to several types of human cancer. In Drosophila, the kinesin-like protein Costal2 (Cos2), which associates directly with the Hh receptor component Smoothened (Smo), is essential for suppression of the transcriptional activity of Ci in the absence of ligand. Another protein, Suppressor of Fused (Su(Fu)), acts in parallel to Cos2 and exerts a weak negative influence on Ci activity. Whereas the loss of Cos2 causes constitutive activation of Hh target genes and is embryonic lethal, Drosophila embryos deficient in Su(Fu) develop into viable and fertile adults. We have found that the Smo C-terminal domain that in Drosophila is phosphorylated in response to Hh and binds to Cos2 is not required for mouse Smo function. Conversely, introduction of the Drosophila Smo C-terminal sequences to mouse Smo renders mammalian Hh signaling Cos2 -sensitive. Furthermore, we show that the two closest mouse orthologs to Cos2, Kif7 and Kif27 fail to affect Shh signaling in mouse cells, and that in contrast, RNAi -induced loss of Su(Fu) expression results in a dramatic increase in Shh pathway. Our results indicate that Drosophila and mammalian Hh signaling mechanisms have diverged at the level of Smo, Cos2 and Su(Fu), and that in mammals, the inhibition of the Hh response pathway in the absence of ligand critically depends on Suppressor of Fused.

810

**Disparate Biological Effects of Intracellular Domains of Notch Ligands: Delta1 Induces p21-dependent Growth Arrest and Jagged1 Induces Apoptosis**

V. Kolev,[1] D. Kacer,[1] M. Duarte,[1] I. Graziani,[1] B. Larman,[1] J. Mitchell,[1] R. Soldi,[1] D. Small,[2] T. Maciag,[1] I. Prudovsky[1]; [1]Maine Medical Center Research Institute, Maine Medical Center, Scarborough, ME, [2]Department of Biochemistry, University of New Hampshire, Durham, NH

Notch signaling plays an important role in the regulation of angiogenesis and endothelial cell phenotype. Activation of Notch receptors results in proteolytical cleavage of their intracellular domains, which translocate to the cell nucleus, and act as transcriptional regulators. Recent findings demonstrated that the Notch ligands, Delta and Jagged, also undergo Notch-dependent cleavage of their intracellular domains. Previously we found that soluble extracellular domains of Delta 1 and Jagged 1 inhibit Notch signaling, and induce substrate-dependent multicellular cord formation. To explore the biological effects of the intracellular domains of Jagged1 and Delta1 in the endothelium, we transduced human umbilical vein endothelial cells with Jagged1 and Delta1 intracellular domains. Cells transduced with beta-galactosidase served as a control. Jagged1 intracellular domain expression induced a p53-independent apoptosis manifested by the exposure of phosphatidylserine on the cell surface, DNA fragmentation, and activation of caspase 3. Conversely, Delta1 intracellular domain expression resulted in a non-proliferating senescent-like phenotype characterized by accumulation of p21 and p27. Further studies revealed that the pro-apoptotic effect of the Jagged 1 intracellular domain and cytostatic effect of Delta 1 intracellular domain were not endothelial-specific but manifested in a wide variety of cell types. Interestingly, p21 knockout but not p27 knockout cells were refractory to the antiproliferative effect of the Delta 1 intracellular domain. Mutation analysis demonstrated that the proliferation blockage by the Delta1 intracellular domain did not require either its nuclear localization or the presence of a C-terminal PDZ-binding domain. Both of the Jagged1 and Delta1 intracellular domain-induced phenotypes were abolished by co-expression of constitutively active Notch1. Our data suggest divergent intracellular roles for Jagged 1 and Delta1 Notch ligands and support the bi-directional model of Notch signaling.

811

**Busulfan-Induced Senescence in WI-38 Fibroblasts**

V. B. Probin, Y. Wang, D. Zhou; Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC

Chronic lung fibrosis induced by various chemotherapeutic agents, such as busulfan (BU), is an important dose-limiting factor for cancer therapy. However, the etiology of chemotherapy-induced lung fibrosis remains to be elucidated. For the first time to our knowledge, we show that WI-38 normal human lung fibroblasts incubated with BU (6.25-125 μM) for 24 hr undergo premature senescence, but not apoptosis, in a dose-independent manner. In contrast, incubation with two other chemotherapeutic agents, etoposide and daunorubicin, induces WI-38 cell apoptosis and/or senescence in a dose-dependent way (high dose-apoptosis and low dose-senescence). Induction of WI-38 cell senescence by a low dose of etoposide (20 μM) is mediated via a well-established DNA damage response pathway: transient activation of the p53-p21 pathway followed by sustained up-regulation of $p16^{Ink4a}$ resulting in a permanent G1 arrest, whereas senescent WI-38 cells induced by BU are permanently arrested in G2 phase in association with a prolonged activation of the Erk (p42/44) pathway and expression of $p16^{Ink4a}$ without significant changes in p53, p38 MAPK and JNK phosphorylation. In addition, inhibition of Erk by a specific inhibitor (PD98059 or U0126) significantly inhibits BU-induced senescence in WI-38 cells. Conversely, inhibition of p38 MAPK, JNK, and p53 with their respective inhibitors has no such effect. Upon removing Erk inhibition, BU-treated cells can proceed to DNA synthesis and cell division. These findings suggest that BU is a distinctive chemotherapy that can cause normal lung fibroblast senescence through a unique mechanism. Induction of fibroblast senescence may contribute to the pathogenesis of lung fibrosis after chemotherapy as senescent fibroblasts produce many soluble molecules and cytokines that are important inflammatory mediators. Therefore, a better understanding of the mechanism of BU-induced lung fibroblast senescence could be beneficial to developing new therapeutic strategies that limit normal tissue damage, thereby improving cancer therapy efficacy.

812

**Blockade of PKC Activation by Nur77 during T Cell Receptor-mediated Apoptosis**

H. Youn, H. Kim, B. Kim; Dept Biochemistry and Molecular Biology, Seoul National University College of Medicine, Seoul, Republic of Korea

TCR engagement is likely to invoke the signaling pathways involved in IL-2 production and Nur77-mediated apoptosis in immature T cells. However, little is known about how TCR decodes two conflicting signaling pathways. Nur77 plays important roles in TCR-mediated apoptosis during negative selection. Here we identified PKC as a novel binding partner of Nur77. Upon TCR stimulation, Nur77 was rapidly translocated into cytoplasm and colocalized with PKC in T cells. Nur77 inhibited the catalytic activity of PKC by direct binding to the ATP-pocket in PKC, which ultimately led to the repression of PKC-targeting transcription factors and endogenous IL-2 synthesis. Moreover, Nur77 induction showed

an inverse relationship with IL-2 synthesis in primary thymocytes. These findings reveal an intrinsic function, namely, that Nur77 represses cytokine production during TCR-mediated apoptosis.

813

**Nucleocytoplasmic Shuttling of Pak5 and its Effects on Apoptosis**

S. Cotteret, J. Chernoff; w451, Fox Chase Cancer Center, Philadelphia, PA

p21-activated kinase 5 (Pak5) is an effector for the small GTPase Cdc42 that is known to activate cell survival signaling pathways. Previously, we have shown that Pak5 is localized primarily to the mitochondria, and that it induces resistance to apoptosis by phosphorylating Bad and perhaps other proteins. To study the relationship between Pak5 localization and its effects on apoptosis, we identified sequence elements that regulate the localization of this kinase. Removal of amino acids 30-83 from the N-terminus substantially decreases the fraction of Pak5 in mitochondria, and increases the fraction of Pak5 in the nucleus. Mutation of a newly-identified nuclear export sequence, comprising residues 401-411, augments this effect, whereas removal of an N-terminal NLS (amino acids 5-10) abolishes it. Blockade of nuclear export with Leptomycin B causes endogenous Pak5 to accumulate in the nucleus, indicating that this kinase shuttles between the nucleus and mitochondria. Pak5 mutants that cannot localize to mitochondria do not confer protection against apoptosis induced by DNA damage. These findings suggest that Pak5 has critical mitochondrial targets that mediate its anti-apoptotic effects. Moreover, reduction of endogenous Pak5 expression in neuroblastoma cells increases their sensitivity to apoptosis. These results show that the subcellular localization of Pak5 is regulated by an interplay between mitochondrial and nuclear targeting motifs, and that its localization is vital to its effects on cell survival.

814

**Biological Effects of Par6 Over-expression in Mammary Epithelia**

M. E. Nolan,[1,2] S. Basu,[1] S. K. Muthuswamy[1]; [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, [2]Genetics Program, Stony Brook University, Stony Brook, NY

Mammary epithelial cells line the ducts of the mammary gland and possess apical-basal asymmetry, referred to as cell polarity. This is essential for epithelial function and it is also thought to play a role in growth control of resting tissue. Recent studies have identified three protein complexes, the Par3/Par6/aPKC complex, Scribble/Lgl/Dlg complex and the Crumbs/PALS1/PATJ complex that act in a hierarchal manner to establish cellular architecture. However, regulation of apical-basal polarity in other contexts such as disease is poorly understood. As loss of epithelial cell architecture and re-initiation of proliferation are common features of early hyperplastic lesions, it is becoming increasingly important to decipher the underlying molecular mechanisms that initiate disruption of apical-basal polarity. Recent evidence suggests that alterations in the polarity machinery may contribute to oncogenesis. Loss of LKB1 (Par4) and Dlg5 are predisposition factors for human cancer and the human papillomavirus protein E6, targets scribble for degradation. It has also been shown that Par6 cooperates with RacV12 to transform fibroblasts. Par6 is thought to play an important role in connecting the structural components of the polarity machinery with cellular signaling pathways. Through its PDZ and CRIB domains it interacts with aPKC, Par3 and CDC42. In addition, Par6 has been shown to interact with Crumbs and Scribble complexes. It is not well understood how these interactions are regulated to provide the multiple functions of Par6. In order to investigate Par6 function in the context of polarized epithelia, we use a non-transformed mammary epithelial cell line (MCF-10A). MCF-10A cells require exogenous EGF for growth and form acini with distinct architecture when cultured on collagen and laminin. We are using both the 3D cell culture system and tradition cell culture to investigate the biological and signaling effects of Par6 overexpression in MCF-10A cells.

815

**Regulation of BAD Serine Phosphorylation by the TWEAK-Fn14 Ligand-Receptor System Induces BCL-XL and BCL-W Protein Expression in Invasive Glioma Cells**

N. L. Tran, S. P. Fortin, B. A. Savitch, W. S. McDonough, M. E. Berens; Neurogenomics, Translational Genomics Research Institute, Phoenix, AZ

Biological hallmarks of glioblastomas multiforme (GBM) include a proclivity for local invasion into the adjacent normal brain tissue and the rarity of systemic metastasis. Invasive GBM cells escape surgery and display resistance to chemotherapeutic- and radiation-induced apoptosis. Recently, we described a transmembrane receptor, fibroblast growth factor-inducible 14 (Fn14), whose level of expression correlates with migrating glioma cells in vitro and with invasive high-grade glioma clinical specimens in vivo. We showed that TWEAK-stimulated glioma cells had increased cellular resistance to cytotoxic therapy-induced apoptosis via NF{kappa}B-mediated up-regulation of BCL-XL and BCL-W mRNA expression (Tran et al. JBC 2005). Here, we show that activation of the TWEAK-Fn14 ligand-receptor system leads to rapid stabilization of BCL-XL and BCL-W proteins via phosphorylation of the anti-apoptotic BAD protein. Phosphorylation of BAD following Fn14 activation occurs on serine 136 and 155 through two independent signaling mechanisms. Fn14-mediated BAD serine 136 phosphorylation is dependent on Akt signaling, whereas, BAD serine 155 phosphorylation is dependent upon p90RSK1 activation. In addition, Fn14 activation induces Erk1/2 phosphorylation in a time-dependent manner; inhibition of Erk1/2 phosphorylation by PD98059 (an inhibitor to upstream MEK1/2 function) suppresses Fn14 induced p90RSK1 phosphorylation. Moreover, depletion of p90RSK1 or inhibition of Akt function by dominant-negative Akt protein results in loss of BAD serine 155 and 136 phosphorylation, respectively, and subsequently enhanced glioma cell sensitivity to cytotoxic-therapy induced apoptosis. We hypothesize that the Fn14 protein functions, in part, to enhance invasive glioblastoma cell survival. Understanding the function and signaling of the TWEAK-Fn14 ligand-receptor system may lead to development of novel therapies to therapeutically target invasive glioma cells.

816

**Signaling Network Measurements as Quantitative Predictors of Cytokine-Induced Cell Death**

S. Gaudet,[1] K. A. Janes,[2] J. G. Albeck,[1] D. A. Lauffenburger,[2] P. K. Sorger[1]; [1]Biology, Massachusetts Institute of Technology, Cambridge, MA, [2]Biological Engineering Division, Massachusetts Institute of Technology, Cambridge, MA

There exists only limited understanding of the strategies cells use to process conflicting cytokine stimuli and regulate responses such as apoptosis. In many cell types, apoptotic responses to cytokines, such as tumor necrosis factor (TNF), can be attenuated by growth factors, such as epidermal growth factor (EGF) and insulin. Network-level understanding of signaling induced by these stimuli requires data on a wide variety of biochemical processes. No single existing technology can gather such a heterogeneous collection of data on a large scale. Using kinase activity assays,

quantitative immunoblots, antibody microarrays, and flow cytometry, we build a systematic signaling dataset that could support the construction of predictive models of signaling networks. Time-dependent signaling profiles were determined in HT-29 human colon carcinoma cells treated with multiple concentrations of TNF in combination with EGF or insulin. The reproducibility, internal consistency, and accuracy of our signaling measurements were validated experimentally to fuse the data into a single self-consistent compendium of over 10,000 cell-signaling and response measurements. Using this compendium, we built partial least squares regression (PLSR) models that predict apoptosis with remarkable accuracy, given signaling data as the input. The accurate predictions of the models allowed us to quantify the information content in the compendium and mine the data for biological insights into the control of apoptotic responses. Our principal finding is that EGF and insulin protect cells from TNF-induced cell death through different means_EGF reduces signaling along a stress-death axis defined by the PLSR model, whereas insulin antagonizes TNF-induced apoptosis by triggering an orthogonal set of prosurvival signals. This work illustrates how large datasets of signals and responses can be analyzed to reveal how protein networks control cellular responses, like apoptosis.

817

**Stereology of the Leydig Cells in Chick Testes during Ontogenic Development**

E. C. Soria,[1] G. Gonzalez-Moran[2]; [1]Patología, Instituto Nacional de Cardiología, Distrito Federal, Mexico, [2]Biologia Comparada, Universidad Nacional Autonoma de México, Distrito Federal, Mexico

The aim of this work was to analyse the ultrastructural changes and variations in the number and size of Leydig cells in testes of *Gallus domesticus* during ontogenic development. Changes were studied on day 8 and 13 of embryonic development, on the day of hatching, 2 and 7 months and 2 years after hatching. Left testes were obtained in all cases, and immediately fixed and embedded in Epon and semi-thick sections were morphometrically measured under light microscopy, using stereologic methods. After that, ultra-thin section of the same material were contrasted with uranyl acetate and lead citrate and examined in a Zeiss EM 10 microscope. Histomorphometric and ultrastructural result indicated that testes of chick from days 8 and 13 of embryonic development show scarse Leydig cells. These cells showed scanty cytoplasm with few lipid droplets and organelles, mainly polyribosomes, rough endoplasmic reticulum cisternae and mitochondria with transversal cristae. In contrast, testes from newly-hatched, immature and aged animals showed numerous Leydig cells arranged in groups or cords characterized by the presence of abundant lipid droplets, mitochondria with tubular cristae and numerous cisternae of smooth endoplasmic reticulum in the cytoplasm. In all these groups of animals there is an increase in the number of Leydig cell, but the size of individual Leydig cells remains relativity constant. In contrast, the increase in volume density of the cords of the Leydig cells in adult chick is due to the enlargement of the individual Leydig cells as compared to the elevation in their cellular number.

818

**Role of TAK1-MKK6-p38 Pathway for NFATc1 Induction and NFκB Transactivation in Osteoclast Differentiation**

H. Huang, J. Ryu, J. Ha, E. Chang, Z. H. Lee, H. Kim; Department of Cellular and Developmental Biology, Institute of Dentistry, College of Dentistry and Brain Korea 21 Program, Seoul National University, Seoul, Republic of Korea

Receptor activator of nuclear factor kappaB (NF-κB) ligand (RANKL) is an essential factor for osteoclast differentiation from bone marrow hematopoietic progenitors. It has been demonstrated that p38 MAPK activity is important for osteoclast differentiation induced by RANKL. In this study we aimed to determine the specific contribution of the signal transduction by this MAPK to osteoclast differentiation. Initially, we investigated the expression levels of signaling molecules lying upstream of p38 MAPK. We found that transforming growth factor-activated kinase 1 (TAK1), MAPK kinase 3 (MKK3), and MKK6 increased by RANKL in early stage of osteoclastogenesis from primary bone marrow cells. This increased expression in p38 upstream components led to enhanced p38 activation. Next, we examined effects of these signaling components on osteoclast differentiation and regulation of NFATc1 and NFκB, transcription factors critically involved in osteoclastogenesis. Retroviral transduction of dominant-negative (DN) forms of TAK1 and MKK6 but not MKK3 reduced osteoclast differentiation. Transduction of TAK1-DN and MKK6-DN and treatment with the p38 inhibitor SB203580 attenuated NFATc1 induction by RANKL. TAK1-DN, MKK6-DN, and SB203580, but not MKK3-DN, also suppressed RANKL stimulation of NFκB transcription activity in p65 phosphorylation-dependent manner without affecting DNA site binding. These results indicate that TAK1 and MKK6 constitute the p38 signaling pathway to participate to osteoclast differentiation through p65 phosphorylation and NFATc1 induction in response to RANKL and that MKK6 and MKK3 have differential roles in osteoclastogenesis from bone marrow precursors.

## Apoptosis I (819-844)

819

**Ionizing Radiation Induces Caspase-Dependent but Chk2- and p53-Independent Apoptosis in Drosophila melanogaster**

A. E. Wichmann,[1] B. R. Jaklevic,[2] T. T. Su[1]; [1]Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO, [2]Molecular and Cell Biology, University of California, Berkeley, CA

In *Drosophila melanogaster*, ionizing radiation (IR) induces apoptosis in the imaginal discs of larvae, typically assayed at 4-6 hours after exposure to a $LD_{50}$ dose. In null mutants of *Drosophila Chk2* or *p53*, apoptosis is severely diminished in these assays, leading to the widely-held belief that IR-induced apoptosis is dependent on *Chk2* and *p53* in *Drosophila*. Here we more closely investigate the dynamics of the apoptotic response and show that IR-induced apoptosis does occur in the imaginal discs of *Chk2* and *p53* mutant larvae, albeit with a significant delay. We demonstrate that this is a true apoptotic response, as it requires caspase activity and the chromosomal locus that encodes the pro-apoptotic genes *reaper*, *hid* and *grim*. We also show that *Chk2*- and *p53*-independent apoptosis is IR-dose dependent and is therefore possibly triggered by a DNA damage signal. We conclude that *Drosophila* has *Chk2*- and *p53*-independent pathways to activate caspases and induce apoptosis in response to IR. This work establishes *Drosophila* as a model for *p53*-independent apoptosis, which becomes an important pathway for inducing cell death in *p53*-deficient cancer cells.

820

**Okadaic Acid Induces a Caspase-Independent Death in Senescent Human Diploid Fibroblasts**

C. P. Davis, K. Hubbard; Department of Biology and The Graduate School and University Center, CUNY Program in Biology, The City College, CUNY, New York, NY

Apoptotic death occurs throughout life. When disrupted cells die prematurely and diseases such as Alzheimer's or Parkinson's result. Certain cancers also arise when cells do not die by apoptosis. Incidentally, Alzheimer's and cancer are prevalent diseases affecting the aging community and understanding the relationship between aging and disease is of importance. For this reason we use senescent human diploid fibroblasts as a model to study aging. Senescent cells have reached their proliferate limit and no longer divide. Gene expression becomes altered, and morphological changes are evident. Due to the changes seen in these cells, we set out to determine whether senescent WI38 fibroblasts can undergo apoptosis and what apoptotic pathway(s) are used. When young, growth-arrested and senescent WI38 fibroblasts are challenged with okadaic acid, senescent WI38's die in a caspase-independent manner whereas initiator caspase-9 and effector caspases-3, -6 are activated in young and $G_0$ cells. Although the caspases are not activated in senescent cells, nuclear substrates specific for caspases are cleaved. DFF45 levels are lowered in senescent cells treated with OA, but the levels remain the same in young and $G_0$ cells. Caspase substrate α-fodrin is cleaved in young, $G_0$ cells but not senescent cells. Propapoptotic Bcl-2 family member Bak was upregulated in all three-growth states, implicating the mitochondrial pathway in apoptotic death. Understanding the apoptotic mechanism enables us understand the reasons for the prevalence of certain diseases in the older population. It may also serve as a means to one day develop effective drugs for the individuals diagnosed with age-related diseases.

821

**Caspase-6 Like Activity in Differentiating Lens Cells is Due to the Proteasome**

A. J. Zandy, S. Bassnett; Ophthalmology, Washington University, St. Louis, MO

During lens fiber cell differentiation, all intracellular organelles including nuclei, mitochondria, endoplasmic reticulum and Golgi are degraded. This provides a transparent fiber cell cytoplasm through which light can pass. The morphological changes that occur during this process are strongly reminiscent of those that occur during programmed cell death, including chromatin condensation and the generation of fragmented DNA with 3'-OH tails. Furthermore, caspase-6-like activity is markedly elevated when organelle breakdown commences (Foley et al. JBC 279(31) 32142-50). Based on these observations, it is widely presumed that the cell death machinery, including the caspase proteases, is responsible for organelle degradation. To test the role of caspases directly, we examined lenses lacking caspase-3, -6 or -7. Contrary to previous reports, we found that these executioner caspases were not required for organelle degradation. Moreover, the rate of cleavage of VEID-AMC (a caspase-6 substrate) was indistinguishable in wildtype and caspase-6 null animals, demonstrating that this activity was not due to caspase-6. We proceeded to identify the lens VEIDase. Using gel filtration, we found that the VEIDase was large (or part of a large (~700 kD) complex). The VEIDase was also inhibited by MG132, NLVS, and clasto-Lactacystin β-lactone, three drugs known to block the proteasome. Finally, immunoprecipitation of the proteasome from lens lysates effectively depleted VEIDase activity. We conclude that the lens VEIDase activity is most likely due to the caspase-like domain of the proteasome. Ongoing studies will evaluate the role of the proteasome in the organelle degradation process.

822

**Reduction of Apoptosis by Differential Inhibition of Caspases - How Xenon Protects Cells**

C. Petzelt; Exp. Anesthesiology, University Hospital Charité, Berlin, Germany

Xenon is probably an ideal neuroprotectant. In view of its outstanding non-toxicity and extraordinary potential for protection it is approaching the gold standard for such application. Up to now, cellular death was measured by the release of lactate dehydrogenase (LDH). Here we show that prevention of activation of late caspases may be the main target for xenon. Apoptosis was induced in rat cortical neurons by hypoxia or staurosporine and LDH release or activation of caspases 1 - 10 was determined. If neurons were pretreated with xenon, cell damage was reduced. Can such a pre-treatment of cells be retained over time? When neurons were treated with xenon for one hour, maintained under normal conditions for various intervals, and then exposed to the insult, cells retained the xenon-effect for up to 4 hours. A similar result was found when cells were post-treated with xenon. By analysing the caspase cascades three groups of caspases could be identified according tho their sensitivity to xenon: The early caspases 8 and 9 are insensitive to xenon. They are activated by the insult and such activation cannot be prevented. The "intermediary" signalling caspases 1,4,5 are reduced in their activation, and the activity of the late the caspases 3 and 7 is almost completely inhibited. These results reinforce the usefulness of xenon for many clinical situations where either an upcoming damage may become probable and therefore justify the preventive use of xenon, or where the damage may have occurred already but where the time window may still allow therapeutic intervention after the insult.

823

**β-lapachone, a DNA Topoisomerase Inhibitor Induces Growth Inhibition and Apoptosis in Bladder Cancer Cells by Modulation of Bcl-2 Family and Activation of Caspases**

J. Lee,[1] D. Choi,[1] H. Chung,[1] H. Seo,[1] Y. Choi[2]; [1]Department of Biochemistry, Busan Science Academy, Pusan, Republic of Korea, [2]Department of Biochemistry, Dong-Eui University College of Oriental Medicine, Pusan, Republic of Korea

β-lapachone is a quinone obtained from the bark of the lapacho tree (*Tabebuia avellanedae*) in South America, which is kwon as a DNA topoisomerase inhibitor. In this study, we report that β-lapachone inhibits the cell growth of human bladder carcinoma cell line T24 and provide a molecular understanding of this effect. The results showed that β-lapachone inhibited the viability of T24 by inducing apoptosis, which could be proved by formation of apoptotic bodies and DNA fragmentation. RT-PCR and immunoblotting results indicated that treatments of cells with β-lapachone resulted in a down-regulation of anti-apoptotic Bcl-2 member and up-regulation of pro-apoptotic Bax expression. β-lapachone-induced apoptosis was also associated with a proteolytic activation of caspase-3 and caspase-9, inhibition of IAP family expression, and degradation of poly (ADP-ribose) polymerase (PARP), phospholipase C-γ1 and β-catenin protein. However, Fas and FasL levels were inhibited by β-lapachone treatment in a concentration-dependent manner. Taken together, our study suggests that β-lapachone-induced apoptosis in T24 cells is mediated, at least in part, by the mitochondrial-signaling pathway.

824

**Lipopolysaccharide Induced Caspase-independent Apoptosis of Human Lung Epithelial Cells**

P. S. Tang,[1,2] M. E. Tsang,[1,2] M. Lodyga,[1,2] X. Bai,[1] A. Miller,[1] B. Han,[1] M. Liu[1,2]; [1]Division of Cellular and Molecular Biology, University Health Network Toronto General Research Institute, Toronto, ON, Canada, [2]Institute of Medical Science, University of Toronto, Toronto, ON, Canada

Caspase-independent cell death has drawn increasing attention. In the present study, we found that LPS accelerated spontaneous death of human lung epithelial A549 cells after they became confluent, in a serum- and cell density-dependent manner. The apoptotic features were demonstrated by TUNEL assay, DNA laddering and Annexin V staining. However, two commonly used pan-caspase inhibitors, zVAD.fmk and BOC-D.fmk, failed to abolish the apoptosis. In contrary, two cathepsin B inhibitors, Ca074-Me or N-1845, reduced cell death significantly. A time-dependent activation of cathepsin B, but not caspase 3, was observed in both control and LPS-treated confluent cells. Although LPS did not further activate cathepsin B or enhance its release from lysosome to cytosol, it induced expression and translocation of apoptosis inducing factor from mitochondria to nucleus as demonstrated by immunofluorescence. LPS-induced apoptosis was significantly attenuated by a free radical scavenger, N-acetyl L-cysteine. Interference of microfilament with cytochalasin D, or lipid raft formation with filipin or methyl-β-cyclodextrin, also reduced apoptosis significantly. These data imply that cells undergo spontaneous cell death mediated by cathepsin B; internalized LPS may trigger release of mitochondrial contents via reactive oxygen species to speed up this caspase-independent apoptosis.

825

**Degradation of Arrestins by Caspases in Apoptotic Cells**

S. KOOK,[1] J. L. Benovic,[2] V. V. Gurevich,[1] E. V. Gurevich[1]; [1]Pharmacology, Vanderbilt University, Nashville, TN, [2]Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

G protein-coupled receptors (GPCRs) activate numerous cellular signaling pathways via their interaction with heterotrimeric G proteins. GPCRs undergo homologous desensitization by a two-step mechanism: phosphorylation of activated receptors by G protein-coupled receptor kinase followed by arrestin binding. Arrestins shield cytoplasmic surface of GPCRs thereby "uncoupling" them from G proteins and effectively terminating G protein-mediated signaling. Although traditionally thought of as mediating GPCR desensitization, arrestins have now been shown to orchestrate GPCR internalization via coated pits and participate in their recycling and also serve as scaffoldings assembling signaling complexes with protein kinases that link GPCRs to alternative G protein-independent signaling pathways. There are two ubiquitously expressed arrestin subtypes, arrestin2 and arrestin3, that interact with numerous GPCRs. In the present study, we demonstrate the cleavage of arrestin2/3 during etoposide-induced apoptosis in Rat-1 cells. Treatment with the caspase inhibitor, zVAD-fmk prevented arrestin2/3 cleavage. Arrestin cleavage occurs relatively early in apoptosis: it is detectable within 6 h and is prominent after 12 h of etoposide treatment. *In vitro* cleavage studies with purified arrestins and recombinant caspases demonstrated that arrestins 2 and 3 are preferentially cleaved by different caspases. In cells, arrestin form complexes with activated phosphorylated GPCRs. In vitro experiments with purified proteins showed receptor binding accelerates the rate and changes the pattern of arrestin2 cleavage, likely due to the well-documented difference in the conformation of free and receptor-bound arrestins. Cell fractionation studies showed that the arrestin2 fragments that are generated by etoposide treatment are localized predominantly to the mitochondria. In addition, overexpression of arrestin2 in Rat-1 cells protected against etoposide-induced apoptosis. These data demonstrate for the first time that arrestins are caspase substrates during apoptosis. Caspase cleavage of free and/or receptor-bound arrestin may represent an important mechanism for the regulation of cell death and survival.

826

**Caspase-Independent Nucleosomal DNA Fragmentation by Endonuclease G under Sustained Endogenous Oxidative Stress**

Y. Ishihara,[1] M. Iwasaki,[1] N. Shimamoto[2]; [1]Department of Biology, Osaka University, Osaka, Japan, [2]Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, Kagawa, Japan

The increased production or decreased elimination of reactive oxygen species inside cells induces oxidative stress, which sometimes causes cell demise, apoptosis or necrosis. We have previously shown that inhibition of both catalase and glutathione peroxidase activities by 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MS) results in endogenous oxidative stress and apoptotic cell death in rat primary hepatocytes, and that caspase family cannot function in this apoptotic cell death because of the oxidized sulfhydryl groups in caspase itself. In addition, the cleavage of inhibitor of caspase-activated deoxyribonuclease (ICAD) was not detected, which indicated no role of caspase-activated deoxyribonuclease (CAD). However, DNA fragmentation was distinctively observed, indicating involvement of other deoxyribonucleases (DNases) than CAD. Therefore, in this study, we investigated DNA fragmentation factor(s) acting on this process of cell death. Pan-DNase inhibitor, aurintricarboxylic acid (ATA) completely inhibited DNA fragmentation induced by treatment with ATZ+MS, showing clear participation of DNase in this DNA fragmentation. Thus, of the DNases we focused on endonuclease G (EndoG), which is reported to act in a caspase-independent manner. Rat EndoG cDNA was cloned from a rat liver cDNA library. Recombinant EndoG activity was inhibited by ATA. However, its activity was not inhibited even at 10 mM hydrogen peroxide. Furthermore, EndoG translocated from mitochondria to nuclei in response to ATZ+MS stimuli. Applying RNA interference method to hepatocytes, mRNA levels of EndoG were almost completely suppressed and the amount of EndoG protein decreased to approximately a half level of untreated cells. Under this condition, DNA fragmentation was significantly suppressed. These results indicate that EndoG was responsible, at least in part, for DNA fragmentation under sustained endogenous oxidative stress conditions induced by treatment with ATZ+MS.

827

**Specific Phosphorylation of c-FLIP$_S$ Determines Its Ubiquitylation and Rapid Turnover**

A. Kaunisto,[1,2] V. Kochin,[1,2] M. Poukkula,[1,2] V. Hietakangas,[1,3] K. Denessiouk,[4] T. Katajamäki,[1,4] M. S. Johnson,[4] L. Sistonen,[1,3] J. E. Eriksson[1,2]; [1]Turku Center for Biotechnology, Turku, Finland, [2]Department of Biology, University of Turku, Turku, Finland, [3]Department of Biology, Åbo Akademi University, Turku, Finland, [4]Department of Biochemistry and Pharmacy, Åbo Akademi University, Turku, Finland

The two c-FLIP proteins, c-FLIP$_S$ and c-FLIP$_L$, modulate death receptor responses by docking to the activated death receptor complex and preventing the formation of active caspase-8 homodimers. The two splice variants have characteristic structural features, with distinct roles in death receptor signaling. Here, we show that both c-FLIP proteins are ubiquitylated and subsequently degraded by the proteasome. However, c-FLIP$_S$ has a dramatically shorter half-life than c-FLIP$_L$, suggesting that distinct mechanisms regulate the turnover of the isoforms. We have identified two

lysines, 192 and 195 (C-terminal to the DED domains), as principal targets for ubiquitylation in c-FLIP$_S$. Surprisingly, the mere presence of these lysines is not a sufficient determinant for their ubiquitylation, as they are found in both isoforms, but ubiquitylated only in c-FLIP$_S$. The efficient ubiquitylation of the target lysines in c-FLIP$_S$ requires the C-terminal splicing tail unique to c-FLIP$_S$. Molecular modeling suggests that the C-terminal tail is crucial for correct positioning and subsequent ubiquitylation of the target lysines. While both c-FLIP proteins are phosphorylated, the phosphorylation of c-FLIP$_S$ is more pronounced. We show that aspartate mutation of a specific phosphorylation site inhibits the ubiquitylation of c-FLIP$_S$, thereby stabilizing the protein. Specific phosphorylation site was confidently determined using thin layer chromatography of *in vivo* $^{32}$P-labeled c-FLIP$_S$ phosphopeptides and MALDI MS. In conclusion, our study reveals isoform-specific control of the ubiquitylation and stability of c-FLIP proteins and shows that the turnover of c-FLIP$_S$ is regulated in a concerted manner by phosphorylation-dependent ubiquitylation. Given the fast turnover of c-FLIP$_S$, this mode of regulation provides a way to rapidly activate or deactivate the inhibitory effect of c-FLIP$_S$ in both apoptotic and inflammatory death receptor signaling.

828

**Rac1 Regulates Apoptosis via N-terminal C-Jun Kinase in IEC-6 Cells**

S. Jin, R. M. Ray, L. R. Johnson; Department of Physiology, University of Tennessee Health Science Center, Memphis, TN

Rac1, a member of the Rho family of small GTPases, is linked to the regulation of the cellular cytoskeleton by inducing the remolding of the actin cytoskeletal structures. Recent evidence suggests that Rac1 also plays an important role in programmed cell death (apoptosis). Previous data from our laboratory led us to investigate the possible involvement of Rac1 in TNF-α-induced apoptosis in intestinal epithelial cells. In the current study, we show that 1) Rac1 is activated by TNF-α within 1 minute and peak level is achieved by 10 minutes and remains elevated for 30 minutes. 2) Inactivation of Rac1 by expression of DN-Rac1 or Rac1 specific inhibitor NSC23766 decreases DNA fragmentation. 3) Inhibition of Rac1 decrease N-terminal c-Jun kinase (JNK) activation. 4) Caspase 3, 8, and 9 activities are decreased in response to Rac1 inhibition. Thus, Rac1 modulates JNK-mediated apoptotic signaling in IEC-6 cells.

829

**Notch Antagonizes the JNK Signaling Pathway by Interfering with the Scaffold Function of JIP1**

J. Kim,[1] M. Kim,[1] K. Kim,[1] H. Yun,[1] J. Chae,[1] S. Hwang,[1] T. Chang,[2] H. Park,[3] K. Lee,[4] P. Han,[4] S. Cho,[5] T. Kim,[6] E. Choi[1]; [1]National Creative Research Initiative Center for Cell Death, School of Life Sciences & Biotechnology, Korea University, Seoul, Republic of Korea, [2]Division of Molecular Life Sciences, Ewha Womans University, Seoul, Republic of Korea, [3]School of Biological Sciences and Technology, Chonnam National University, Kwangju, Republic of Korea, [4]Department of Neuroscience, Ewha Womans University, Seoul, Republic of Korea, [5]Department of Animal Biotechnology, Konkuk University, Seoul, Republic of Korea, [6]Department of Pathology, Columbia University, College of Physicians and Surgeons, Seoul, Republic of Korea

The transmembrane protein Notch is cleaved by γ-secretase to yield an active form, Notch intracellular domain (Notch-IC), in response to the binding of ligands such as Jagged. Notch-IC contributes to the regulation of a variety of cellular events including cell fate determination during embryonic development as well as cell growth, differentiation and survival. We now show that the SAPK/JNK pathway was suppressed by overexpression of the murine Notch-IC (mNotch-IC). Ectopically expressed Numb, a negative regulator of Notch, blocked Notch1-IC-mediated inhibition of JNK activation. Ectopic expression of mNotch-IC in MEF$^{PS1(-/-)}$ cells suppressed $H_2O_2$-stimulated JNK activity and apoptosis. Notch1-IC also inhibited both JNK activation and apoptosis triggered by glucose deprivation. Taken together, these findings suggest that Notch1-IC functions as a negative regulator of signaling by the c-Jun N-terminal kinase pathway.

830

**Activation of JNK3 after Injury Induces Cytochrome C Release by Facilitating Pin1-Dependent Degradation of Mcl-1**

Q. Li,[1] C. Tep,[1] T. Yune,[1] X. Zhou,[2] K. Lu,[2] S. Yoon[1]; [1]Center for Molecular Neurobiology, Ohio State University, Columbus, OH, [2]Medicine, Cancer Biology Program, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

Evidence has indicated that the JNK pathway plays a role in neuronal survival, but its mechanism remained unclear. We report that a selective increase in JNK3 activity after spinal cord injury is responsible for oligodendrocyte apoptosis. Upon activation, JNK3 induces cytochrome C release in part by facilitating degradation of Mcl-1 whose stability is normally maintained by Pin1. In the basal state, we found that Mcl-1 is phosphorylated at p-Thr163Pro, most likely by ERK, resulting in Pin1 recruitment. With injury, JNK3 phosphorylates Mcl-1 at Ser121, displacing Pin1, thereby facilitating degradation of Mcl-1 via ubiquitination. These data together suggest that JNK3 induces degradation of Mcl-1 by disrupting its association with Pin1. This report thus reveals a mechanism by which JNK3 regulates cytochrome C release, thereby regulates neuronal apoptosis under pathological conditions.

831

**Src Regulates Apoptosis in IEC-6 Cells by Activating STAT3 and Akt**

S. Bhattacharya, R. M. Ray, L. R. Johnson; Department of Physiology, University of Tennessee Health Science Center, Memphis, TN

Intestinal epithelial (IEC-6) cells are resistant to apoptosis following the inhibition of ornithine decarboxylase (ODC) and subsequent depletion of polyamines. The depletion of polyamines rapidly activates NF-κB and STAT3, which is responsible for the observed decrease in apoptosis. Since both NF-κB and STAT3 signaling pathways can be activated by Src kinase, we examined its role in the antiapoptotic response. Inhibition of ODC by α-difluoromethylornithine increased the activity of Src within 30 min. Activation was prevented by exogenous putrescine added to the DFMO containing medium. Inhibition of Src with PP2 and a dominant negative Src construct prevented the activation of Akt, JAK and STAT3. Spontaneous apoptosis was increased in cells in which Src was inhibited and the protective effect of polyamine depletion was lost. Our results suggest that the inhibition of ODC rapidly removes a small pool of available polyamines triggering the activation of Src which in turn activates Akt and JAK resulting in the translocation of NF-κB and STAT3 to the nucleus and the subsequent synthesis of antiapoptotic proteins.

832

**Studies on the Modulation of p53 and p21 Expression and the Enhancement of UVB Protection in Human Cells Through Stress-Free Hsp70 Induction by Artemia Extract**

C. Dal Farra, K. Cucumel, É. Bauza, N. Domloge; Vincience, Sophia antipolis, France

Corresponding studies of our own have demonstrated that Artemia extract 3% induces Hsp70 in human cells in a stress-free manner. Based on this data, we were interested in studying the protection from UVB insult offered by this stress-free induction of Hsp70, in cultured human fibroblasts and ex vivo skin organ culture. Cells were exposed to different UV doses after Hsp70 induction. Immunoblotting studies revealed that at low UVB doses, p53 and p21 were up-regulated more than in control cells, which suggests an enhancement in cell cycle arrest and in the action of the DNA repair pathway. These findings were confirmed by the comet assay which showed, no UVB-induced DNA degradation in Artemia-induced Hsp cells. Interestingly, at higher doses of UVB, our results were similar and showed that in Artemia-induced Hsp cells, both p53 and p21 levels were higher than in control cells. A significant decrease in cell damage and apoptosis was also seen in Artemia-induced Hsp cells at higher doses of UVB, which strongly suggests the action of the repair pathway rather than the apoptosis pathway, while most of the control cells showed the activation of p53-dependent apoptosis. In order to confirm this result, Hsp70 was induced in a similar manner in ex vivo skin prior to UVB exposure. In contrast to the extended signs of UV damage exhibited by the control skin, treated skin samples exhibited a great conservation of structure. Moreover, immunostaining of Langerhans cells revealed no signs of UV-induced immunosuppression in the Artemia-induced Hsp70 skin samples. These studies demonstrate that stress-free Hsp70 induction by Artemia extract is an approach that can be of great use in improving cell defense against UV stress.

833

**A Redox-based Mechanism of Growth Arrest and Induction of Apoptosis in Response to Cytotoxic Agents**

T. De Luca,[1] D. M. Morre,[2] D. Morre[1]; [1]Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, [2]Foods and Nutrition, Purdue University, West Lafayette, IN

Our work has identified an NADH oxidase with protein disulfide-thiol interchange activity, designated tNOX, that is associated with unregulated growth of cancer cells and response to chemotherapeutic drugs. Substances that block tNOX include adriamycin, cis-platinum, the isoflavene phenoxodiol, the green tea catechin (-)-epigallocatechin-3-gallate and the vanilloid capsaicin. All result in growth arrest and induction of apoptosis accompanied by an increase in ceramide and a decrease in the prosurvival signaling molecule, sphingosine-1-phosphate (S1P). Implicated are activation of sphingomyelinase (SMase) to generate ceramide and inhibition of sphingosine kinase (SphK) to lower S1P. Our hypothesis is that one or more of the immediate products resulting from drug inhibition of tNOX would be responsible for SMase activation and SphK inhibition. When tNOX is inhibited, reduced coenzyme Q accumulates in the plasma membrane and NADH accumulates at the cytosolic plasma membrane surface. Treatment of recombinant neutral sphingomyelinase (nSMase) with reduced glutathione blocks activity, whereas NADH stimulates. If a normal function of the protein disulfide-thiol interchange activity of tNOX is to keep nSMase reduced, inhibition of tNOX would result in nSMase auto-oxidation and subsequent release from inhibition. These two changes, nSMase oxidation and NADH accumulation in the vicinity of the nSMase at the plasma membrane appear to be sufficient to account for nSMase activation and ceramide accumulation preceding growth arrest. In contrast, SphK, also located at the cytosolic side of the plasma membrane, is markedly inhibited by NADH in HeLa cells. Thus, inhibition of plasma membrane electron transport and attendant increased cytosolic NADH appear sufficient to initiate both growth arrest and apoptosis by cancer cell-specific cytotoxic agents via increased ceramide and the FAS-dependent apoptotic cascade.

834

**Lithium Blocks Ceramide- and Etopside-Induced Apoptosis via a Mechanism of Inhibiting Protein Phosphatase 2A Methylation and Activation**

C. Chen,[1] C. Lin,[1] C. Chiang,[2] M. Jan,[3] Y. Lin[1]; [1]Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan Republic of China, [2]Molecular Medicine, National Cheng Kung University Medical College, Tainan, Taiwan Republic of China, [3]Microbiology and Immunology, Chung-Shan Medical University, Taichung, Taiwan Republic of China

Lithium confers cell protection against stress and toxic stimuli. Although lithium inhibits a number of enzymes, the anti-apoptotic mechanisms of lithium remain unresolved. Here we report a novel role of lithium on the blockage of ceramide- and etoposide-induced apoptosis via inhibition of protein phosphatase 2A (PP2A) activity. During ceramide- and etoposide-induced apoptosis, lithium abolished the reduction of mitochondrial transmembrane potential and caspase-9 and -3 activation. PP2A inhibitor, okadaic acid (OA), caused an effect similar to that of lithium. Ceramide- and etoposide-activated caspase-2 and -8 was also blocked by lithium and OA. Lithium- and OA-mediated inhibitory effects were not observed after cytochrome *c* had been released, indicating that lithium and OA both acted upstream of mitochondria. Overexpression of PP2A resulted in caspase-2 activation, mitochondrial damage, and cell apoptosis that were inhibited by OA and lithium. Ceramide- and etoposide-induced Bcl-2 dephosphorylation at serine 70 was abrogated by lithium and OA. Furthermore, ceramide- and etoposide-induced PP2A activation involved methylation of PP2A C subunit, which lithium suppressed. Lithium caused dissociation of the PP2A B subunit from the PP2A core enzyme, whereas ceramide caused recruitment of the B subunit. Taken together, lithium exhibited an anti-apoptotic effect by inhibiting Bcl-2 dephosphorylation and caspase-2 activation upstream of mitochondrial damage, which involved a mechanism of downregulating PP2A methylation and PP2A activity.

835

**Glycogen Synthase Kinase-3β Modulates Ceramide-Induced T Cell Mitochondrial Apoptosis**

C. Lin,[1] C. Chen,[1,2] C. Chiang,[3] M. Jan,[4] Y. Lin[1]; [1]Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan Republic of China, [2]Institute of Basic Medical Sciences, National Cheng Kung University Medical College, Tainan, Taiwan Republic of China, [3]Institute of Molecular Medicine, National Cheng Kung University Medical College, Tainan, Taiwan Republic of China, [4]Department of Microbiology and Immunology, Chung-Shan Medical University, Taichung, Taiwan Republic of China

The signaling of glycogen synthase kinase-3β (GSK-3β) has been implicated in stress-induced apoptosis. However, the pro-apoptotic role of GSK-3β remains unclear. Here we show the involvement of GSK-3β in ceramide-induced T cell mitochondrial apoptosis. First, ceramide-induced GSK-

3β activation by protein dephosphorylation at serine 9 was demonstrated. GSK-3β inhibitors including lithium chloride and SB216763 blocked the activation of GSK-3β and cell apoptosis caused by ceramide. Also, ceramide-induced caspase-2 and -8 activation, Bid cleavage, mitochondrial transmembrane potential reduction, cytochrome *c* release, and activation of caspase-9 and -3 were abolished by GSK-3β inactivation. Similarly, blockage on the ceramide-induced mitochondrial apoptosis was detected in cells with GSK-3β silencing using short interfering RNA technique. Furthermore, protein phosphatase 2A (PP2A)-regulated phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB) signaling was involved in GSK-3β activation by ceramide. These findings indicate the dependence of GSK-3β regulation in ceramide-induced T cell mitochondrial apoptosis through PP2A- and PI3K/PKB-mediated signaling.

836

**A Novel Marker (PC-cholesterol Complex) for Foam Cell Apoptosis Can Be Induced by Hyperlipidemic Serum**

M. Mori,[1] H. Ogasawara,[1] A. Tanaka,[1] R. Soshi,[1] A. Isomura,[1] S. Kagiwada,[1] T. Asano,[1] H. Itabe,[2] T. Takano[1]; [1]Molecular Pathology, Fac. of Pharm.Sci. Teikyo University, Kanagawa, Japan, [2]Biochemistry, Fac. of Pharm.Sci. Showa University, Tokyo, Japan

**[Objective]** Phosphatidylcholine (PC)-cholesterol (FC) complex structures, a unique atheroma related antigen recognized by a novel monoclonal antibody ASH1a/256C. To elucidate the role of antigen in the development of atherosclerosis, the relationship among PC-FC complex, FC increase and apoptotic events in cultured foam cell were investigated. **[Method]** J774 cells incubated in the presence of hyperlipidemic sera or modified lipoproteins for 24 hr, then cultured up to 3-13 days. PC-FC complex were detected by immunofluorescent microscopy. UPR marker (CHOP, XBP-1) was detected by immunoblot analysis. **[Results & Discussion]** Using human or WHHL rabbit hyperlipidemic sera and J774 cells, we succeeded to express PC-FC complex on the surface of FC-rich lipid droplets as a foam cell death marker. PC-FC complex could be formed only when the cells were incubated with hyperlipidemic sera or AcLDL, not VLDL, LDL, moreover, VLDL enhances PC-FC complex formation by AcLDL; indeed, VLDL could not induce it by itself. Annexin-V positive cells were observed at day 3 induced by hyperlipidemic sera, whereas native LDL, HDL and VLDL failed to induce among FC-rich lipid droplets, PC-FC complex and Annexin-V binding structures. TUNEL positive cells were also observed in this foam cell culture at day 4 or later, whereas, no apoptotic cells were observed after treatment with U18666A in order to block cellular cholesterol transport. The UPR reaction marker, CHOP and XBP-1 were also detected. However, some HMG-CoA reductase inhibitors especially vascular-Statin (Simvastatin and Atorvastatin) were able to block the foam cell apoptosis not through FC induced ER stress. The hydrolysis and rearrangement of cellular cholesterol take place in foam cells to form the antigen; there were significantly relationship between the PC-FC complex formation and foam cell death.

837

**Electrapoptosis: Ultrashort Electrical Pulses Stimulate Skin Tumors to Self-Destruct**

R. Nuccitelli,[1] U. Pliquett,[1] W. Ford,[1] X. Chen,[1] J. Swanson,[1] S. J. Beebe,[2] J. F. Kolb,[1] K. H. Schoenbach[1]; [1]Center for Bioelectrics, Old Dominion University, Norfolk, VA, [2]Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, VA

We have been using a new technology applying ultrashort electric pulses to treat melanoma skin tumors in mice. These pulses are so fast that charges on the tumor cell membranes do not have time to redistribute and screen out the imposed electric field from the cell interior (50 ns rise time). Therefore, these pulses penetrate into every cellular organelle for the duration of the pulse. These nanosecond pulses are very different from classical electroporation pulses that are much longer and have rise times in the microsecond domain, thereby preventing penetration of the electric field into the cell interior. Using nanosecond pulses with amplitudes greater than or equal to 2 volts/μm, a single treatment of 100 pulses causes the melanoma tumors to shrink by as much as 90% within about 10 days. Two targets of these pulses are the capillaries feeding the tumor and the tumor cell nuclei. Capillaries feeding the tumor become leaky immediately following the pulsed field application, disrupting the blood supply to the tumor. Red blood cells leak out and are dispersed around the tumor but blood flow to the tumor is not detectable by either transillumination or ultrasound Doppler imaging. In addition to the reduction in blood flow, tumor cell nuclei shrink by 40% and become pyknotic within an hour after pulsing. The threshold field for this response generates a two-volt difference across an organelle that is one μm in diameter, which is sufficient to cause organelle membrane breakdown or nanopore formation for the duration of the pulse. For a 100-pulse treatment, the total time that the tumor cells are exposed to this field is 0.03 ms, 100 times shorter than a single action potential in a nerve or muscle.

838

**17β-Estradiol and an ERα Agonist (PPT) Trigger Apoptosis in Human Adrenal Carcinoma Cells but an ERβ Agonist (DPN) Does Not**

J. W. Brown, L. M. Prieto, C. Perez-Stable, L. M. Fishman; Endocrinology Research, Department of Medicine, VA Medical Center, University of Miami Miller School of Medicine, Miami, FL

Earlier studies have shown that high-dose (10-5 M) 17β-estradiol (E) treatment has a G2/M blocking effect in SW-13 human adrenal carcinoma cultures and is a strong trigger of apoptosis. To examine the differential effects of estrogen α and β receptors (ER α and β) in this system, we incubated SW-13 cells with the specific ER α and β agonists (PPT [4,4',4''-(propyl-[1H]-pyrazole-1,3,5-triyl)-trisphenol] and DPN [2,3-bis(4-hydroxy-phenyl)-propionitrile], respectively; 10-5 M). We then used flow-cytometry to analyze the percentage of cells in various phases of the cell cycle (apoptosis, G1, S and G2/M). E treatment for 48-hrs increased apoptosis more than 5-fold (from 3.6 ± 0.5 % to 20 ± 2.2 % of cells; $p<0.01$). PPT had a similar effect, increasing apoptosis 6-fold to 22 ± 1.7 % ($p< 0.01$) but DPN caused no change (3.9 ± 0.8 % vs. 3.6 ± 0.5 %). E was found to block in G2/M, increasing it from 15 ± 0.4 % to 21 ± 1.0 % ($p<0.01$), but neither PPT nor DPN had any effect. Both E and PPT decreased the percentage of cells in G1 (from 59 ± 1.4 % for control to 34 ± 2.3 % for E and 40 ± 2.0 % for PPT; $p< 0.01$), while DPN had no effect. Neither E, PPT nor DPN affected cells in S phase. These studies suggest that induction of apoptosis by E in SW-13 cultures is likely mediated by the ER α receptor. Experimental results also imply that the G2/M blocking effect of E is not due to either ER α or β mechanisms. These studies were supported by the U.S. Department of Veterans Affairs and the South Florida Veterans Affairs Foundation for Research and Education.

839

**Over Expression of Human ClpP Reduces AIF Levels and Inhibits Cisplatin-Induced Apoptosis in Huh4 Cells**

Y. Zhang,[1] V. Barr,[2] M. R. Maurizi[1]; [1]Lab of Cell Biology, NCI/NIH, Bethesda, MD, [2]Lab of Cell Molecular Biology, NCI/NIH, Bethesda, MD

ClpXP is a bipartite chaperone/protease machine that catalyzes ATP-dependent protein unfolding and degradation in bacteria and in subcellular

compartments of eukaryotes. The biological functions of the ClpXP in mammalian cells have not been characterized. We investigated the effects of human ClpP (hClpP) over expression on cisplatin-induced apoptosis in the human hepatoma cell line, huh-4. We found that over expression of hClpP in huh-4 cells inhibits cisplatin-induced apoptosis. High levels of hClpP caused a 50-55% reduction in the number of cells undergoing apoptosis between 16-48 h following cisplatin treatment. A corresponding decrease in amounts of activated PARP and caspases 3, 7, and 9 was observed in the cells over expressing hClpP. Interestingly, the basal level of apoptosis inducing factor (AIF) was significantly reduced in hClpP cells prior to cisplatin treatment. Consequently, the release of AIF from mitochondria to the cytosol following cisplatin treatment was much lower in cells over expressing hClpP. High-level expression of hClpP did not affect the total amount or release of cytochrome-c, Smac, or HTRA2/OMI. Taken together, these results suggest that hClpP exerts its effect on cisplatin-induced apoptosis by modulating the intracellular levels of AIF possibly by reducing AIF synthesis or increasing its degradation. Our results indicate that the apoptotic response in cells will be affected by conditions that increase or decrease the intracellular levels of pro- or anti-apoptotic proteins. Current work is focused on the mechanism of the hClpP-mediated effects on AIF expression, which might provide insight into the biological functions of ClpXP in mammalian cells.

840

**Shiga Toxin 1 is Internalized and Causes Apoptosis in Gb3-negative Intestinal Epithelial Cells**

M. S. Laiko,[1] M. Donowitz,[1] A. Kane,[2] O. Kovbasnjuk[1]; [1]Johns Hopkins University School of Medicine, Baltimore, MD, [2]GRASP Center, Tufts New England Medical Center, Boston, MA

The foodborne illnesses caused by the Shiga toxin 1 (Stx1) producing enterohemorrhagic *Escherichia coli* (EHEC) include bloody diarrhea, hemolytic uremic syndrome (HUS) and encephalopathy. The current view is that Stx1 binding to its receptor the glycosphingolipid $Gb_3$ is the initial step in a cascade leading to HUS. However, it has recently been shown that human colonic epithelium does not express Gb3. The goal of our research was to characterize the mechanisms of interaction of Stx1 and its recombinant B-subunit (Stx1B) with Gb3-negative intestinal epithelial T-84 cells and compared it to the Stx1/Stx1B effects on Gb3-positive Caco-2 cells. Confocal microscopy shows that Stx1B-Alexa 488 interacts with both cell types. In Caco-2 cells Stx1B is taken up through the retrograde pathway into the Golgi/ER. In T84 cells Stx1B appears in intracellular vesicles that co-localize with 40K dextran, but not in Golgi/ER. Western immunoblotting shows that Stx1 and Stx1B induce apoptosis in both CaCo-2 and T-84 cells. However, the apoptotic mechanisms triggered by holotoxin vs. B-subunit are different. Exposure of CaCo-2 and T-84 cells to Stx1 for 72 hours increased intracytosolic cytochrome C and AIF, activated caspase-3 with PARP cleavage. In contrast, Stx1B didn't induce caspase-3 cleavage in T-84 or CaCo-2 cells. It released mitochondrial cytochrome C and activated caspase-2 in both cell types. We conclude that Stx1/Stx1B is internalized by Gb3-free intestinal epithelial cells, which triggers the apoptosis. This shows the presence of receptor-independent uptake of Stx1 that may be important for intestinal and systemic disease. .

841

**What Effects do Household Chemicals, Permethrin and Propylene Glycol, Have on U937 Cells and A549 Cells?**

T. N. Ezell,[1] E. Benjamin,[2] Y. Hijji,[2] L. Taylor[1]; [1]Biology, Morgan State University, Baltimore, MD, [2]Chemistry, Morgan State University, Baltimore, MD

Multiple chemical sensitivity (MCS) syndrome occurs with low-level chemical exposure. People are continuously exposed to natural and synthetic chemicals. Many of these chemicals have the ability to cause acute and chronic inflammatory responses. Studies have estimated that approximately 64,000 people in the United States die prematurely from heart and lung disease every year due to pollution and chemical exposures. Permethrin (PTN) and propylene glycol (PG) are synthetic chemicals that are found in many household products such as cosmetics, deodorants, lotions, insect foggers, flea dips, garden and turf products, mosquito sprays, termite treatments, and agricultural products. Previous studies have determined that PG and PTN at low doses decrease immune cell recognition and responses to foreign antigens. At high doses PTN is also know to cause inflammation and necrosis. The present study explores the mechanism of action of PTN and PG by measuring alterations in cellular growth, viability, and toxicity. U937 and A549 cells were cultured in 24-well plates at 0.5-1 X $10^5$ cells/well for 2 hours. The cells were then exposed to 0 - 10 µg/ml of PG and/or 0 - 0.5 ng/ml of PTN. The cultures were incubated for 24, 48, and 72 hrs at 37$^o$C and 5% $CO_2$. At each time point, the cells were observed, counted, stained with Calcein and Ethidium Homodimer-1 and photographed using fluorescence and light microscopy. Preliminary data suggest that U937 cells and A549 cells exposed to PTN, PG and PTN + PG exhibited a dose dependent decrease in cellular growth.G12RR017581P, DOE grant DE-FG0T-03ER63580

842

**Apoptosis Regulates Notochord Development in *Xenopus laevis* Embryos**

M. A. Malikova, M. Van Stry, K. Symes; Department of Biochemistry, Boston University School of Medicine, Boston, MA

The notochord is a defining characteristic of the chordate embryo. It develops at the midline during gastrulation and persists as a long, flexible rod that underlies the neural tube and extends through most of the length of the embryo. The notochord plays crucial roles throughout embryonic development, such as inducing ectoderm, endoderm, and mesoderm derivatives in the early embryo, structurally, as a primitive skeleton, and ultimately forming the intervertebral discs of the spine. Our recent evidence in *Xenopus* suggests that notochord development is regulated, at least in part by apoptosis. We find that TUNEL positive cells appear in the notochord beginning at stage 18 and that the number of dying cells increases with developmental stage. In addition, these apoptotic cells are distributed in an anterior to posterior pattern that is correlated with notochord extension through vacuolization. We do not find any TUNEL positive cells in the somitic mesoderm at the stages examined (stages 15-25). Inhibition of notochord cell death by overexpression of the anti-apoptotic factor, Bcl-2, causes an increase in the length-to-width ratio of mesoderm explants and the notochord is approximately double the length of that in control explants. In intact embryos, Bcl-2 overexpression causes axial defects such as spina bifida. The length of the notochord in these embryos, however, is not increased, but the notochord is severely buckled. This buckling also appears in an anterior to posterior pattern with developmental stage. We propose that during normal development, to coordinate the extension rate of the notochord during vacuolization with surrounding tissues such as the somites and neural tube, cells are removed from the notochord by apoptosis.

843

**Slug (Snail2) Is an Antiapoptotic Factor during Tubulogenesis of MDCK Cells**

P. Leroy, K. E. Mostov; Anatomy, UCSF, San Francisco, CA

Slug (Snail2), a member of the Snail family of transcription factors, has been described as a key regulator of epithelial-mesenchymal transition (EMT) and a repressor of E-cadherin. Slug has also been involved in processes that do not required a full EMT and in the protection of cells from cell death. Here, we present evidences that Slug is induced during Hepatocyte Growth Factor (HGF)-induced tubulogenesis of Madin-Darby Canine Kidney (MDCK) cells and that it has an antiapoptotic role during this process. Tubulogenesis is a process involved in the formation and maintenance of diverse organs, during which cells undergo important morphogenetic modifications requiring tight control. Epithelial MDCK cells are able to form tubules in a 3-dimensional culture system. MDCK cells seeded in a collagen gel form cysts which are spherical monolayers of cells enclosing a central lumen. Within 24hrs of HGF treatment, the cysts form extensions and chains and after 2 to 3 days, tubules. The first phase of extension and chain is described as a partial EMT (p-EMT) during which the cells lose their epithelial polarity but not their epithelial markers. We show that Slug is upregulated by HGF during the p-EMT phase of tubulogenesis and that its expression decreases during tubule formation. The upregulation of Slug is not accompanied by a down-regulation of E-cadherin expression. Both the MEK inhibitor U0126 and the PI3K inhibitor LY294002 block Slug upregulation independently. Using an inducible antisense Slug construct to block the expression of Slug, we found that Slug knockdown does not impair the initial phase of extension but leads to apoptosis of the cells that have undergo extension. This work reports for the first time the involvement of Slug during MDCK tubulogenesis and links its role with an EMT-dependent process and apoptosis.

844

**Differential Apoptosis Regulation Following Changes in Bcl-2's Sub-cellular Localization**

B. P. Portier, G. Taglialatela; University of Texas Medical Branch, Galveston, TX

B-cell leukemia/lymphoma 2 (Bcl-2) is the prototypical member of a large family of proteins that function as modulators of apoptosis. While Bcl-2 functions as an anti-apoptotic protein when localized at the mitochondria its function at other organelles (ER and nucleus) is largely unreported. Here we used an *in vitro* model to investigate nuclear localized Bcl-2's role in promoting/inhibiting apoptosis. Our results show rapid Bcl-2 expression (via transient transfection) leads to increased nuclear localized Bcl-2 in PC12 cells. However, nuclear localization is lost following long term (stable) Bcl-2 expression. Using Invitrogen's pGeneSwitch inducible expression system to express Bcl-2/YFP (a Bcl-2 yellow fluorescent fusion protein) paired with flow cytometry, we are able to specifically investigate nuclear associated Bcl-2's role in apoptosis. Using flow cytometry on whole cells and isolated nuclei following induced Bcl-2/YFP expression allows direct and simultaneous measurement of apoptotic status, Bcl-2 expression and nuclear localization. It is our hypothesis that high Bcl-2 expression leads to nuclear association and if prolonged in time, apoptosis. In addition, co-expression of FKBP38 can effectively prevent Bcl-2 from associating with the nuclear compartment. These studies should further our understanding of Bcl-2's function at different sub-cellular locations. These data also suggest a novel involvement of Bcl-2 functioning as a pro-apoptotic protein, a characteristic that could lead to new strategies aimed at blocking Bcl-2 induced apoptosis thereby protecting cells undergoing apoptotic stress. ACKN: NINDS Pre Doctoral Fellowship 1 F30 NS49759-01A1

## Mitosis & Meiosis II (845-868)

845

**Identification and Characterization of New Drosophila Mitotic Genes by Co-expression Analysis and RNA-mediated Interference**

M. P. Somma,[1] E. Bucciarelli,[1] V. Naim,[1] F. Ceprani,[1] V. De Arcangelis,[1] R. Piergentili,[1] A. Palena,[1] G. Belloni,[1] L. Ciapponi,[1] R. Petrucci,[1] G. Cenci,[1] M. G. Giansanti,[1] F. Vernì,[1] C. Pellacani,[1] B. Fasulo,[1] F. Di Cunto,[2] M. Goldberg,[3] M. Gatti[1]; [1]Dip. Genetica e Biologia Molecolare, Istituto di Biologia e Patologia Molecolari del CNR, Rome, Italy, [2]Dipartimento di Genetica, Biologia e Biochimica, Università di Torino, Torino, Italy, [3]Department of Molecular Biology and Genetics, Cornell University,, Ithaca, 14853-2703, NY

Based on the principle that genes involved in the same biological process tend to be significantly co-expressed, we exploited the extant microarray data to order the Drosophila genes according to their co-expression with known mitotic genes. We verified that the first 1000 genes of our co-expression list include more than one half of the known mitotic functions. With the aim of identifying novel genes involved in mitotic cell division, we performed RNAi experiments to inactivate each of these 1000 genes. Drosophila S2 cells were treated with double stranded RNA for three days and then analyzed for the normality of the chromosomes and the mitotic spindle. This screen led to identification of over 70 genes that have not previously been implicated in mitosis. Ablation of these genes resulted in a variety of mitotic phenotypes: (a) chromosome breakage, (b) abnormal chromosome condensation, (c) precocious sister chromatid separation, (d) defective spindle assembly, (e) metaphase arrest, (f) abnormal chromosome segregation, and (g) failures in cytokinesis. Most of the Drosophila genes identified in our screen have homologues in other eukaryotes including humans, thus providing a putative function for a fraction of the human genome.

846

**Regulation of Klp61F by *Drosophila* Wee1**

K. Garcia,[1] J. Stumpff,[2] T. Su[1]; [1]Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO, [2]Physiology and Biophysics, University of Washington, Seattle, WA

Wee1 tyrosine kinases act during interphase to phosphorylate and inactivate cyclin dependent kinase1 (Cdk1), thereby preventing entry into mitosis. Our observations, however, indicate that *Drosophila* Wee1 (dWee1) also plays a role in mitotic spindle function that is independent of its role in Cdk1 regulation at mitotic entry. We find that *dwee1* mutant embryos display disrupted mid-spindles, microtubule spurs extending from the mitotic spindle, and collisions between adjacent spindles. In addition, we found that dWee1 physically interacts with Klp61F, the *Drosophila* homolog of the BimC/Eg5 kinesin that is involved in mitotic spindle organization and centrosome separation. Klp61F displays decreased tyrosine phosphorylation in extracts from *dwee1* mutant embryos, and dWee1 directly phosphorylates Klp61F on tyrosine residues *in vitro*. Mass spectrometric analyses identified four phosphorylated peptides in Klp61F after incubation with dWee1. Three peptides fall within the motor domain and the remaining peptide is part of the BimC box located in the tail domain. Since these domains are important for Klp61F function, our objective is to show that dWee1 has a role in mitotic spindle function by regulating Klp61F via phosphorylation. Our future plans include the

determination of the functional importance of *dwee1*-dependent phosphorylation on Klp61F.

847

**The Kinesin-6, PavKLP, Mediates Spindle-Cortical Interactions in *Drosophila* Embryos**

P. Sommi, M. Kwon, S. Morale-Mulia, D. Cheerambathur, A. Mogilner, J. M. Scholey; Center for Genetics and Development, University of California, Davis, CA

The morphogenesis of the mitotic spindle is a dynamic process that is known to be driven by microtubules (MTs) and associated molecular motors but recent studies suggest that cortical organization also plays a key role in this process. Here we examined the contribution of the kinesin-6 family member, PavKLP to mitotic spindle and cortical dynamics in *Drosophila* embryos. Using a newly-generated anti-PavKLP antibody for hydrodynamic studies, we determined that PavKLP behaves as a dimer in embryonic extracts. Immunofluorescence revealed that PavKLP associates with puncta on mitotic spindles and on the cortex. Cortical PavKLP punctae specifically co-localize with actin at the mitotic furrows from interphase through anaphase and the functional disruption of PavKLP by antibody microinjection causes defects in spindle pole separation, central spindle organization and the formation and ingression of actin furrows. In addition we tested the hypothesis that PavKLP may contribute to furrow dynamics by driving vesicle transport. Using immunofluorescence, we observed that PavKLP co-localizes with Golgi vesicles localized at the embryonic cortex and in aster-like MT structures. In S2 cells, where PavKLP depletion by RNAi leads to defects in both central spindle organization and cytokinesis, Golgi and PavKLP co-localization was confirmed from interphase through cytokinesis. Based on the disorganization of actin following PavKLP inhibition and the co-localization of PavKLP and Golgi vesicles, we propose that PavKLP mediates spindle-cortical interactions by transporting vesicles along astral MTs to the cortex and is thus required for the proper organization of cortical furrows during mitosis.

848

**Role of *Drosophila* Pericentrin-like Protein (D-PLP) in Centrosome Maturation**

E. P. Lucas, N. Peel, T. Barros, J. W. Raff; Wellcome Trust/CR-UK Gurdon Institute, Cambridge, United Kingdom

The *Drosophila* Pericentrin-like protein (D-PLP) is the only recognisable protein with a Pericentrin-AKAP450 centrosomal targeting (PACT) domain in flies. D-PLP is a large coiled-coil protein that is concentrated in both the centrioles and the PCM throughout the cell cycle. Initial studies in larval brain cells suggested that this protein was required for the efficient centrosomal recruitment of γ-tubulin and of all the PCM components tested (CNN, D-TACC, Msps, CP190 and CP60), as their centrosomal localisation was strongly disrupted in *d-plp* mutant cells. Surprisingly, however, all the PCM components tested could eventually be recruited to centrosomes in at least some *d-plp* mutant cells, and mitosis was not dramatically perturbed. Therefore, in order to further study the potential role of D-PLP in recruiting and tethering γ-tubulin and other PCM components to the centrosome, we are currently investigating how the PCM components can be recruited to the centrosomes in the absence of D-PLP. Our data from *d-plp* germline clone analysis revealed that D-PLP is not required for the recruitment of PCM components to the centrosomes, but instead, it seems to play an important role in maintaining the structural integrity of the PCM. In addition, it also suggests that there is some other force that holds the PCM together in the absence of D-PLP and microtubules. Moreover, we are also investigating whether there is a functional redundant mechanism that can maintain the structural integrity of the centrosomes whenever D-PLP is absent.

849

**Chromokinesins Affect the Flux Rate of Mitotic Spindle Microtubules in *Drosophila* S2 Cells**

D. W. Buster, D. J. Sharp; Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY

Chromokinesins, a family of chromosome-associated microtubule motors, are potential generators of aneuploidy since they are believed to participate in spindle morphogenesis and chromosome movements during mitosis. Previously, we have reported that knock-down of some chromokinesins in cultured *Drosophila* S2 cells leads to a significant increase in chromatid number per cell, though not all chromokinesins have identical affects, suggesting that they perform different functions in chromosome segregation. In addition, some chromokinesins significantly increase the mitotic index and the frequency of multi-nucleation. To examine possible mechanisms that could require chromokinesin activity, we are testing the hypothesis that chromokinesins affect the flux rate of spindle microtubules. Single (or combinations of) chromokinesins are knocked-down by RNA interference in S2 cells expressing a low level of GFP-tubulin, and the flux rates of the mitotic spindle microtubules are measured by fluorsecent speckle microscopy. Interestingly, our results suggest that RNAi of different chromokinesins can have opposite affects on flux rates. RNAi of the chromokinesin, KLP38B, increases the flux rate, while RNAi of a putative chromokinesin, KLP88A, produces a trend of flux rate reduction. Therefore, chromokinesins can impact flux rates, but their affects are not identical. Unexpectedly, some chromokinesins (eg, KLP38B, nod) might impose a drag on fluxing microtubules in vivo.

850

**The CLASP, Mast, and Microtubule-depolymerizing Kinesins Interact Functionally to Regulate Spindle Length and Flux in *Drosophila* S2 Cells**

D. W. Buster, D. J. Sharp; Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY

Proper mitotic spindle formation and chromosome segregation in higher eukaryotes require that spindle microtubules flux at normal rates. Recently, two spindle-associated proteins, KLP10A and Mast/Orbit, were found to strongly impact flux rates in different systems. To study the functional interplay of these flux generators, we used RNA interference (RNAi) to knock-down these proteins individually or in combination. The short spindle phenotype generated by Mast RNAi was rescued by co-RNAi of KLP10A, but was also partly rescued by co-RNAi of either KLP59C or KLP67A, two centromeric kinesins. Flux rates of spindle microtubules were significantly decreased by either KLP10A or Mast RNAi, but the Mast RNAi affect could be partly rescued by co-RNAi of KLP59C or KLP67A, but not KLP10A. Therefore, KLP10A and Mast have balanced but opposing effects on spindle lengths, but both activities are required for normal flux rates.

851

**Unique Requirement for MCAK in Microtubule Disassembly During Spindle Morphogenesis**

R. Ohi,[1] M. L. Coughlin,[1] T. J. Mitchison[1,2]; [1]Systems Biology, Harvard Medical School, Boston, MA, [2]Institute for Chemistry and Cell Biology,

Boston, MA
Bipolar meiotic spindle assembly in *Xenopus* extracts depends on microtubule (MT) depolymerization by the type 13 kinesin MCAK and its regulation by the Aurora B kinase. This mechanism stimulates chromatin-driven MT assembly and is complementary to the Ran pathway, which activates MT assembly promoting factors. KIF2 is a second Kinesin 13 present in this system suggesting complexity in MT depolymerization control during chromosome segregation. To understand tasks unique to MCAK and KIF2, as well as their potential functional redundancies, we first established using quantitative microtubule depolymerization assays that MCAK and KIF2 are comparatively potent microtubule destabilizing enzymes *in vitro.* KIF2 is also similar to MCAK in that its MT depolymerizing activity is inhibited by Aurora B-dependent phosphorylation. Despite these similarities, we find that perturbation or removal of KIF2 from *Xenopus* egg extracts has no consequence on spindle assembly, indicating that MCAK is uniquely required for this process. Notably, KIF2 is ~4-fold less abundant than MCAK in *Xenopus* extracts, suggesting that the dominant role of MCAK in spindle assembly may simply reflect its greater protein concentration. This is not the case, however, as replacement of MCAK with KIF2 results in spindle assembly failure and large cytoplasmic radial MT asters. We speculate that KIF2 is only critical for MT depolymerization control at inner centromeres, whereas MCAK is required both for cytoplasmic MT dynamics regulation as well as MT dynamics regulation within the meiotic spindle.

852

**Analysis of *Xenopus* Xorbit/CLASP in Spindle Assembly and Function**

E. Hannak, R. Heald; MCB, UC Berkeley, Berkeley, CA
A family of non-motor microtubule binding proteins, Orbit/MAST/CLASP has emerged as an important player during mitosis, functioning in chromosome congression and maintenance of spindle bipolarity in *Drosophila* and mammalian cells. Here, we used meiotic egg extracts to gain insight into the role of the *Xenopus laevis* homologue, Xorbit, in spindle assembly and function. By immunofluorescence, Xorbit localized along the length of spindle microtubules, concentrating at kinetochores on metaphase spindles and shifting to the spindle midzone in late anaphase. Immunodepletion of Xorbit or its inhibition by addition of a dominant negative fragment, GST-CT, prevented chromosome alignment at the metaphase plate and resulted in aberrant microtubule structures including monopolar and short spindles. We have identified a biochemical interaction between Xorbit and the kinetochore-associated kinesin CENP-E, which may contribute to the role of Xorbit in chromosome congression. Xorbit-depleted extracts failed to form bipolar structures around chromatin-coated beads in the absence of centrosomes and kinetochores, indicating that it also plays an important role in chromatin-driven microtubule polymerization. The stabilizing role of Xorbit was most apparent during anaphase, when spindle microtubules depolymerized rapidly upon Xorbit inhibition. These data reveal a crucial role for Xorbit in stabilizing both kinetochore and non-kinetochore microtubules. We propose that Xorbit tethers dynamic microtubule plus ends to kinetochores and chromatin, providing a stabilizing activity essential for spindle assembly and chromosome segregation.

853

**Dynein Light Intermediate Chain 1 Has a Unique Function in the Spindle Assembly Checkpoint**

T. L. Wadzinski, S. J. Doxsey; Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA
The spindle assembly checkpoint (SAC) ensures that a cell has bi-oriented chromosomes that accurately divide between daughter cells. This checkpoint has two components. First, Mad2 localizes to kinetochores until adequate microtubule attachment is achieved. Second, Bub1 and BubR1 localize to kinetochores until there is adequate tension across them. Dynein is a kinetochore associated protein complex that induces a metaphase arrest by activating the SAC when disrupted. This complex is implicated in moving Mad2 from kinetochores and generating tension across kinetochores. Light intermediate chain 1 (LIC1) is a poorly understood component of dynein. Here we show that RNAi-mediated depletion of hLIC1 in human cells results in a metaphase arrest; timing of the other mitotic phases is unaltered. This arrest is not due to a general dynein disruption because in hLIC1 depleted cells dynein functions normally in golgi and spindle organization, dynein localizes normally to kinetochores and spindle poles, and the dynein complex is intact. In fact hLIC1 depleted metaphase cells have fully congressed chromosomes that appear to be under tension as Bub1 and BubR1 are gone from kinetochores. Mad2 localization remains unclear however Mad2 depletion allows hLIC1 depleted cells to go through mitosis, implicating the SAC in this arrest. Cdc2 phosphorylation and regulation of LIC1 in mitosis has been reported previously. Interestingly LIC1 phosphorylation status plays a role in this checkpoint because expressing phosphomemetic forms of rat LIC1 allow hLIC1 depleted cells to get through the checkpoint whereas pseudo-unphosphorylated rLIC1 does not relieve the checkpoint. We are using this unique phosphorylation status in a proteomic approach to identify interacting proteins involved in this checkpoint. We propose that LIC1 links Mad2 to dynein and without LIC1, dynein cannot move Mad2 off the kinetochore to relieve the SAC.

854

**Rapid Microtubule Dynamics in *Drosophil*a Embryonic Mitotic Spindles**

D. Cheerambathur, G. Civelekoglu-Scholey, I. Brust-Mascher, P. Sommi, A. Mogilner, J. M. Scholey; Center for Genetics and Development, University of California, Davis, Davis, CA
Spindle microtubules are highly dynamic and are measured to turnover with characteristic times of the order of 10 to 100s of seconds in most organisms. Here we used Fluorescence Recovery After Photobleaching (FRAP) of GFP-tubulin in *Drosophila* embryos to observe microtubule dynamics within the mitotic spindle and show that the turn-over rate of MTs in *Drosophila* spindles is very high, with a half time for recovery of 5-7 sec (Brust-Mascher et al. 2004). Our recent study shows, surprisingly, that this rapid recovery half time is the same irrespective of the size and the region of bleach zone and regardless of the mitotic stage when spindle bleaching is performed, from metaphase till telophase. This fast turn over rate is slowed down by the inhibition of *Drosophila* EB1, a protein that has been shown to affect dynamic instability parameters of MTs. Currently we are investigating several mutants of other proteins which functions as dynamic instability regulators to test their effect on the recovery rate. We are complementing these studies with computer simulations based on a previously developed model (Brust-Mascher et al. 2004) to investigate the distributions of MT plus and minus ends and MT lengths that could give rise to such rapid recovery rates and match the change in the recovery rate in response to drugs that alter MT dynamics.

855

## Integrin Signaling and Control of Spindle Orientation

F. Toyoshima, E. Nishida; Cell and Developmental Biology, Kyoto University, Kyoto, Japan

Alignment of the mitotic spindle with a predetermined axis occurs in many types of polarized cells such as cells in epithelial tissues, but it has been unknown whether there is a mechanism regulating spindle orientation in non-polarized cells. We found that integrin-dependent cell adhesion to the substrate orientates the mitotic spindle of non-polarized cultured cells, such as HeLa cells, parallel to the substrate plane in a cytoskeleton-dependent manner. The spindle is properly oriented in cells plated on fibronectin or collagen, but not in cells on poly-L-lysine. Moreover, the spindle is misoriented in cells treated with the RGD peptide or anti-integrin antibody, indicating requirement of integrin-mediated cell adhesion for this mechanism. This integrin-dependent spindle orientation is independent of gravitation or cell-cell adhesion, but requires actin cytoskeleton, astral microtubules, microtubule plus-end-tracking protein EB1, and myosin X. We are now analyzing roles for membrane components, which are known to be involved in integrin signaling, in the control of spindle orientation.

856

## Mechanism Controlling Perpendicular Alignment of the Spindle to the Axis of Cell Division in Fission Yeast

Y. Gachet,[1] C. Reyes,[1] S. Goldstone,[1] J. Hyams,[2] S. Tournier[1]; [1]LBCMCP-CNRS UMR5088, Institut d'Exploration Fonctionelle des Génomes (IFR109), Université Paul Sabatier, Toulouse, France, [2]Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand

In animal cells, the mitotic spindle is aligned perpendicular to the axis of cell division. This ensures that sister chromatids are separated to opposite sides of the cytokinetic actomyosin ring (CAR). We show that, in fission yeast, spindle rotation is dependent on the interaction of astral microtubules with the cortical actin cytoskeleton. Interaction initially occurs with a region surrounding the nucleus, which we term the astral microtubule interaction zone (AMIZ). Simultaneous contact of astral microtubules from both poles with the AMIZ directs spindle rotation and this requires both actin and two type V myosins, Myo51 and Myo52. Astral microtubules from one pole only then contact the CAR, which is located at the centre of the AMIZ. Finally, we show that the position of the mitotic spindle is monitored by a checkpoint that regulates the timing of sister chromatid separation. We find that whereas sister kinetochore pairs normally congress to the spindle midzone before anaphase onset, this congression is disrupted when astral microtubule contact with the actin cytoskeleton is disturbed. By analyzing the timing of kinetochore separation, we find that this anaphase delay requires the Bub3, Mad3, and Bub1 but not the Mad1 or Mad2 spindle assembly checkpoint proteins. In agreement with this, we find that Bub1 remains associated with kinetochores when spindles are mispositioned. These data indicate that, in fission yeast, astral microtubule contact with the medial cell cortex is monitored by a subset of spindle assembly checkpoint proteins.

857

## INT6 and Moe1 Interact with Cdc48 to Regulate Mitotic Progression in *Schizosaccharomyces pombe*

J. H. Otero,[1] E. C. Chang[2]; [1]Interdepartmental Program in Cell and Molecular Biology, Baylor College of Medicine, Houston, TX, [2]Department of Molecular and Cellular Biology Breast Cancer Center, Baylor College of Medicine, Houston, TX

*INT6* is a key gene involved in the formation of breast cancer, as it is a frequent site of integration by the mouse mammary tumor virus. To better understand the functions of Int6, we have characterized an Int6 homolog (Yin6) in the fission yeast *Schizosaccharomyces pombe.* Our data support a model in which Yin6 regulates many mitotic functions, including spindle formation, chromosome segregation, and mitotic exit by controlling the localization and assembly of subunits of the proteasome. In my study, I plan to further characterize Yin6 by focusing on a evolutionarily conserved protein, Moe1, to which Yin6 binds directly. In two-hybrid screens, we have isolated several proteins that are involved in the endoplasmic reticulum associated degradation (ERAD) pathway as Moe1-binding proteins. Of these, we are particularly interested in Cdc48, which like Yin6 and Moe1, regulates proteasome mediated protein degradation and spindle dynamics. In support of the two-hybrid data, our biochemical data show that Cdc48 binds Yin6 and Moe1 in *S. pombe* cells. Furthermore, genetic analyses show that mutations affecting *cdc48,* together with mutations inactivating either *yin6* or *moe1,* acts synergistically to create a severe mitotic defect. These observations support the hypothesis that the Yin6-Moe1 complex can interact with Cdc48 to regulate mitosis and whether their actions converge upon proteolysis regulation is currently under investigation.

858

## Correlating *HZW10* Functional Domains to Mitotic Checkpoint Function

J. K. Famulski,[1] G. K. Chan[1,2]; [1]Oncology, University of Alberta, Edmonton, AB, Canada, [2]Experimental Oncology, Cross Cancer Institute, Edmonton, AB, Canada

Human *Zeste White* (hZW10) is an essential mitotic checkpoint protein involved in ensuring proper chromosome segregation during mitosis (Chan *et al.* 2000). hZW10 localizes to the kinetochores during prometaphase where it forms a conserved complex with *Rough Deal* (hRod) (Scaerou *et al.* 2001). Recruitment of hZW10 to the kinetochore has been shown to be hZwint dependent (Wang *et al* 2004). The hZW10/hRod complex is responsible for the recruitment of the microtubule motor, dynein/dynactin and checkpoint proteins Mad1 and Mad2 to the kinetochore (Chan *et al.* 2000; Kopps *et al.* 2005). We have performed a structure function study of hZW10 to determine its function(s) within the mitotic checkpoint. Using random 5 amino acid insertion mutagenesis, deletions and truncations, we have generated mutants of hZW10 with disrupted functional domains. So far we have identified mutants with disrupted kinetochore localization, dynamitin and hZwint interaction domains as assayed by immunofluorescence and yeast two hybrid analysis. Contrary to published results, we found that the C-terminus of hZW10 is required for kinetochore localization but dispensable for hZwint interaction. Furthermore, a N-terminal 52 amino acid deletion in hZW10 prevents hZwint interaction but still retains kinetochore localization. This result suggests that hZwint is necessary but not sufficient for hZW10 kinetochore localization. Disruption of hZW10-dynamitin interaction has no effect on hZW10 kinetochore localization. Dimerization of hZW10 might be a possible explanation for our results. We are examining the hZW10 mutants for dominant negative phenotypes *in vivo*. Mitotic checkpoint function is assayed by monitoring mitotic escape in the presence of vinblastine. We are examining the disruption of kinetochore microtubule attachment by immunofluorescence. We are also examining the changes in hZW10 dynamics at kinetochores by Fluorescence Recovery After Photobleaching (FRAP). Our assays will correlate distinct functional domains of hZW10 to mitotic checkpoint function.

859

**The Chromosomal Passenger Complex Regulates HP1 Localization by Phosphorylation of Histone H3 Serine 10**

B. Tseng, H. Funabiki; Laboratory of Chromosome and Cell Biology, The Rockefeller University, New York, NY

The functional significance of histone H3 serine 10 phosphorylation during M phase is unclear. Fischle, Wang, and Allis have proposed the binary switch hypothesis, which states that phosphorylation of histone H3 serine 10 inhibits the binding of heterochromatin protein 1 (HP1) to the adjacent trimethylated lysine 9 of histone H3 (H3K9me3)[1]. Since Aurora B, a member of the chromosomal passenger complex (CPC), phosphorylates histone H3 serine 10, we examined whether this complex regulates the chromosomal binding of HP1 via this phosphorylation using *Xenopus* egg extracts. We found that although depletion of the CPC did not affect H3K9me3 levels, both endogenous xHP1α and exogenous xHP1α, β, and γ bound better to metaphase chromosomes assembled in extract depleted of the CPC than in control extract. The dependence of this enhanced association on H3K9me3 was verified by two methods. First, alanine mutation in xHP1α at tryptophan 57, which is important for the binding to H3K9me, diminished the chromosomal binding of xHP1α in the absence of the CPC. Second, the addition of an H3K9me3 peptide, but not an unmodified H3 peptide, to CPC-depleted extracts reduced the chromosomal association of xHP1α-GFP. These data are consistent with the binary switch hypothesis and suggest that one of the outcomes of histone H3 serine 10 phosphorylation during M phase by the CPC is dissociation of HP1 from chromosomes. 1. W. Fischle, Y. Wang, C. D. Allis, *Nature* **425**, 475 (2003).

860

**Structural Analysis of the Interaction Between the Cohesin Complex and Cohesin Attachment Regions**

A. Surcel,[1] R. T. Simpson[2]; [1]Huck Institute for the Life Sciences, Pennsylvania State University, University Park, PA, [2]Biochemistry and Molecular Biology Department, Pennsylvania State University, University Park, PA

Sister chromatid cohesion is facilitated by the multimeric protein complex known as cohesin. From late G1 phase to mitotic metaphase, the cohesin complex binds to specific sequences along chromosome arms identified in *Saccharomyces cerevisiae* as Cohesin Attachment Regions (CARs). These DNA sequences are interspersed on average by 9kb and show no sequence similarity to each other. This lack of sequence homology, coupled with cohesin dependence on the chromatin remodeling factor RSC, suggests that the underlying chromatin structure of these loci may be important. There are currently three main models for cohesin binding - the embrace model, the snap model and the physical contact model. To determine the feasibility of these models, we are using the Minichromosome Affinity Purification (MAP) method to investigate the ultrafine structure of CARs when bound by cohesin. Isolated minichromosomes are analyzed by transmission electron microscopy. Our data indicates that this method is a viable option for assessing model accuracy of cohesin maintenance at CAR loci. We are also performing large-scale, low-resolution nucleosome mapping of CARs and cohesin loading loci to determine whether chromatin structural changes occur concurrently with cohesin binding or loading. Our preliminary data suggests that scc2/4 binding sites - the places of cohesin loading - undergo nucleosome protection during S-phase, implying that a changing chromatin structure may be important for sister chromatid cohesion.

861

**The Human Mitotic Checkpoint Protein BubR1 Regulates Chromosome-Spindle Attachments**

M. A. Lampson, T. M. Kapoor; Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY

Loss or gain of whole chromosomes, the form of chromosomal instability most commonly associated with human cancers, is expected to arise from the failure to accurately segregate chromosomes in mitosis. The mitotic checkpoint is one pathway that prevents segregation errors by blocking anaphase onset until all chromosomes make proper attachments to the spindle. Another process that prevents errors is stabilization and destabilization of connections between chromosomes and spindle microtubules. An outstanding question is how these two pathways are coordinated to ensure accurate chromosome segregation. We find that in human cells depleted of BubR1, a critical component of the mitotic checkpoint that can directly regulate anaphase onset, chromosomes do not form stable attachments to spindle microtubules, as indicated by several independent assays. Kinetochores of these cells lack cold-stable microtubule fibers and contain high levels of Mad2 and the dynactin component p150. Stable attachments are restored in BubR1-depleted cells by Aurora kinase inhibition, which is known to stabilize kinetochore-microtubule attachments. Loss of BubR1 function thus perturbs regulation of attachments rather than the ability of kinetochores to bind microtubules. Consistent with this finding, depletion of BubR1 increases phosphorylation of CENP-A, a kinetochore-specific Aurora kinase substrate. We propose that BubR1 links regulation of chromosome-spindle attachment to mitotic checkpoint signaling.

862

**Localization of Emi1 to the Spindle Poles Spatially Restrains APC/C Activity to Maintain Spindle-associated Cyclin B and Spindle Assembly**

K. Ban,[1] J. Torres,[2] J. Miller,[2] M. Nachury,[2] P. Jackson[2]; [1]Program in Cancer Biology, Stanford University, Stanford, CA, [2]Pathology, Stanford University, Stanford, CA

Cell cycle progression depends on the ordered and timely destruction of substrates by the anaphase-promoting complex (APC/C), an E3 ligase that targets substrates for ubiquitination. Emi1 inhibits the APC/C during S and G2 to allow the accumulation of cell cycle substrates. In early mitosis, the bulk of Emi1 is destroyed, permitting the destruction of a subset of substrates such as cyclin A. In contrast, mitotic substrates such as cyclin B and securin are not destroyed until anaphase. This stability is due in part to the inhibition of the APC/C by the spindle checkpoint, but it is unclear how additional mechanisms help to restrain APC/C activity while checkpoint signals are established. Here, we show that a population of Emi1 localizes to the spindle poles and provides spatial regulation of APC/C activity important for proper spindle assembly. Using immunofluorescence, we demonstrate that the localization of Emi1 to the poles occurs at nuclear envelope breakdown and requires microtubules and the minus-end directed dynein-dynactin motor complex. Purification of the Emi1 complex further identified the spindle pole organizer NuMA together with components of the APC/C, suggesting a localized regulatory network at the mitotic spindle poles. Consistent with this idea, siRNA knockdown of Emi1 in mitotic cells leads to reduction of spindle associated cyclin B on the spindle causing mislocalization of spindle organizing proteins NuMA/dynactin and formation of aberrant spindles that fail to congress chromosomes. Similarly, immunodepletion of Emi1 from *in-vitro* aster assembly reactions causes premature loss of cyclin B/cdk1 activity and inability to organize microtubules into asters. Together these findings support a model that localization of Emi1 to the poles spatially restrains APC/C activity to maintain spindle-associated cyclin B important for pole

integrity and spindle assembly.

863

**Centromere-specific Role of the Cohesin Complex in *Xenopus* Egg Extract**

R. Deehan Kenney, R. Heald; UC Berkeley, Berkeley, CA

Cell propagation requires accurate replication and segregation of the genetic material. To facilitate their accurate distribution by the mitotic spindle, sister chromatids are tethered during DNA replication and simultaneously released at the metaphase to anaphase transition, allowing for their separation to opposite spindle poles. The highly conserved, multi-subunit cohesin complex is fundamental to this process, yet its role in mitosis is not fully understood. To characterize cohesin function, we immunodepleted the complex from *Xenopus laevis* egg extracts. Loss of cohesin resulted in unpaired sister chromatids that failed to align on the metaphase plate, and aberrant chromatid distribution during anaphase. Topologically linking metaphase chromosomes by inhibiting topoisomerase II (topo II) partially rescued metaphase compaction and alignment defects but did not restore sister chromatid cohesion. Chromosomal passenger proteins Incenp and Aurora B did not target to centromeres properly in the absence of cohesion, however, this could be rescued by addition of monastrol, thereby relieving bipolar spindle tension on the kinetochores. The dynamic redistribution of passenger proteins to the midzone during anaphase was unaffected. When both condensin and cohesion were immunodepleted, we observed an additive effect on spindle assembly, and a rescue of the kinetochore stretching caused by condensin depletion. These data underscore the essential requirement for cohesin in sister chromatid cohesion, targeting of passenger proteins and kinetochore function.

864

**Histone Depletion Causes Altered Centromere Clustering and Spindle Structure in Budding Yeast**

D. C. Bouck, K. Bloom; Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Prior to anaphase onset, sister chromatid pairs align along the metaphase plate of the mitotic spindle. During this step of mitosis, the mitotic spindle is stabilized by interdigitated interpolar microtubules, while kinetochore microtubules exert poleward force on chromosomes at kinetochore-centromere attachment sites. The poleward pulling force is opposed by cohesion along the arms of the sister chromatids. The balance of these opposing forces results in an equilibrium where centromeres remain fairly organized along the metaphase plate. The contributions of microtubule dynamic instability, motor proteins, microtubule-associated proteins, kinetochores, and cohesion complexes in this process have been previously explored and remain under active investigation. In this study, we have undertaken the analysis of mitotic spindle structure during metaphase in cells where chromatin structure has been altered. By downregulating the transcription of histone genes, we have perturbed a key member of the metaphase spindle, thus allowing us to better understand the contribution of chromatin as a structural element. Using budding yeast strains where histone H3 or H4 transcription is under the repressible galactose promoter, we have lowered the concentration of nucleosomes in the cell and examined the effects on mitotic spindle structure. Spindles are longer after histone depletion, suggesting that spindle length is physically restrained by the inward force due to chromosome arm cohesion. We have also found that centromeres are de-clustered during metaphase after histone depletion. This might be caused by the loss of persistent microtubule-kinetochore attachment, or variability in kinetochore microtubule length. These results indicate that chromatin packaging is a significant factor in the alignment of sister chromatids in metaphase and contributes to the overall structure of the mitotic spindle.

865

**Requirement of Plus-end Microtubule Dynamics for Proper Spindle Pole Assembly at the Centrosomes**

J. Hornick, J. Breunig, K. T. Vaughan, E. H. Hinchcliffe; Biological Sciences, Univ. Notre Dame, Notre Dame, IN

The poles of the mitotic spindle are assembled by the pair of centrosomes, which recruit structural proteins, such as NuMA, to the minus ends of microtubules (MTs). Dynamic MTs are necessary for proper formation of mitotic spindle poles; NuMA is transported to the spindle pole in a dynein-dependant fashion, which itself requires dynamic MT plus-ends for “search-capture”. To investigate the mechanisms of spindle pole assembly, BSC-1 cells constitutively expressing either GFP-α tubulin or GFP-NuMA were continuously treated with μM concentrations of Taxol to completely suppress plus-end microtubule dynamics, and then imaged by time-lapse fluorescence video microscopy as they entered mitosis. At the onset of mitosis, the microtubule network in Taxol-treated cells dissociates from the centrosome, and microtubules are recruited to the nuclear envelope and the cell cortex. The duplicated centrosomes often fail to separate, do not remain attached to the nucleus, and fail to assemble prominent microtubule asters. A significant number of centrosomes do not have any microtubules associated with them. In addition, suppressing microtubule dynamics leads to significant delays in nuclear envelope breakdown, resulting in NuMA “leaking” out of the nucleus, and moving predominately to the cortex, rather than to the centrosomes. Unexpectedly, the cortically arrayed NuMA and microtubules bud off into multiple cytasters, which migrate, cluster and fuse together around the chromosomes, but fail to assemble a proper bipolar microtubule array. This ultimately leads to failure of cytokinesis, and the generation of a polyploid cell. Our data are consistent with the model that plus-end microtubule dynamics are required for search-capture during prophase to properly localize NuMA, thereby ensuring that the spindle poles assemble only at the centrosomes. Our live-cell imaging has also directly revealed the cellular mechanism for the anti-mitotic activity of Taxol.

866

**Defective Centrosomal and Spindle Functions after Protein 41R Downregulation**

J. R. Spence,[1] M. Go,[1] S. Bahmanyar,[2] A. Barth,[2] W. J. Nelson,[2] S. W. Krauss[1]; [1]Genome Biology, UC-LBNL, Berkeley, CA, [2]Molecular and Cellular Physiology, Stanford University School of Medicine, Palo Alto, CA

Multifunctional structural proteins, serving as adaptors or linkers, are essential for cellular remodeling processes. Protein 4.1R was initially characterized as a crucial ~80kD structural component of mature red cells with well-defined functions stabilizing interactions within spectrin-actin lattices and cytoplasmic domains of transmembrane proteins. In nucleated cells, we previously characterized 4.1 as an integral “core” centrosome component, colocalizing with centriolar tubulin, in the surrounding pericentriolar matrix, and on fibers connecting centriolar pairs. As multifunctional structural proteins, centrosomal 4.1 isoforms could serve as structural elements linking components to impart dynamic properties and fidelity needed for centrosomal functions. Using an open-cell *Xenopus* extract system, we showed that 4.1 is essential for spindle and centrosome assembly and for regulation of microtubule dynamics and organization. Current investigations in mammalian cells include both 4.1R

(red cell) and another family member, 4.1G (generally distributed). Using 4.1 gene-specific antibodies we observed that both 4.1R and 4.1G localize at centrosomes but have differential subcentrosomal distributions during the cell cycle. To probe 4.1R function, we specifically downregulated 4.1R expression by RNAi and characterized a unique phenotype relating to centrosomal dysfunction: (1) interphasic cells with disorganized tubulin not emanating from a centrosomal focus and multinucleated cells often containing disparate-sized nuclei; (2) perturbed ninein distribution at mature centrioles; (3) decreased frequency of mature centriole separation preceding entry into mitosis; (4) monopolar and multipolar mitotic spindles with misaligned decondensed chromatin, disorganized microtubules and mislocalized NuMA; (5) defective cytokinesis including chromosome missegregation, spindle dysmorphology, and improper tubulin bridge formation. These data suggest that 4.1R, acting as an adaptor or linker to multiple mitotic targets, could affect cell division by regulating the architectural integrity of cell division machinery. Based on our data we are analyzing 4.1 interactions impacting microtubule nucleation/anchoring, chromatin condensation and motor-dependent microtubule organization.

867

**Human Sgo1 is Essential for the Maintenance of Centromeric Cohesion of Sister Chromatids During Mitosis**

X. Wang, Y. Yang, W. Dai; Medicine, New York Medical College, Valhalla, NY

Human Sgo1 is essential for the maintenance of sister chromatid cohesion Xiaoxing Wang, Yali Yang, Wei Dai, New York Medical College, Valhalla, NY 10595 Sister chromatid cohesion relies primarily on the cohesin complex, which consists of Smc1, Smc3, Scc1 (Rad21), and Scc3. Cohesin forms a ring structure that may help to entrap DNA molecules. In high eukaryotes such as mammals, cohesin molecules are dissociated from chromosomal arms but not from centromeres during early mitosis by the so-called “prophase pathway”. Although the molecular basis by which centromeric cohesin is retained remains unclear, recent studies have shed light on an evolutionarily conserved protein called Shogoshin (Sgo) that may explain the delayed removal of centromeric cohesin until the anaphase onset. In HeLa cells, a major fraction of Sgo1 is localized to kinetochores during prophase and metaphase, during which it is phosphorylated and exhibits a significant mobility shift on SDS gels. Suppression of Sgo1 via RNA interference causes massive mitotic arrest and chromosome mis-segregation. Mitotic arrest induced by Sgo1 depletion is accompanied by partial inactivation of BubR1 and Mps1, two major spindle checkpoint components, whereas no significant change of cohesin levels is detected. Moreover, phospho-MEK and phospho-ERK levels are significantly increased in mitotic cells induced by Sgo1 depletion but not by nocodazole treatment. Our studies thus indicate that Sgo1 plays a critical role in mediating integrity of kinetochores during mitosis and that dysregulated function of Sgo1 may be one of the major underlying causes for chromosomal instability and aneuploidy.

868

***Drosophila* Myb is Required for Centromere Maintenance**

J. Manak,[1] S. Heidmann,[2] D. Georlette,[3] T. Van,[1] J. Lipsick[1]; [1]Pathology and Genetics, Stanford University, Stanford, CA, [2]Genetics, University of Bayreuth, Bayreuth, Germany, [3]Molecular and Cell Biology, University of California, Berkeley, CA

In most eukaryotic cells, centromeres are essential for chromosome segregation. CID/CENP-A, a centromere-specific histone H3 variant, is required to localize other centromere-binding proteins. However, little is known about the deposition and maintenance of CID itself. DmMYB, the Drosophila homologue of the MYB oncoprotein family, is required for organismal viability and genomic stability. Previous work has shown that DmMYB localizes to newly replicated DNA and is required for the switch from genome-wide DNA replication to site-specific chorion gene amplification in endocycling ovarian follicle cells. Diploid, mitotically cycling cells can proliferate in the absence of DmMYB, but eventually display an increased frequency of arrest with partially condensed chromosomes as well as defects in chromosome segregation. We now show that DmMYB is required for the proper maintenance of CID at the centromere. In addition, DmMYB is necessary for the non-centromeric deposition of overproduced CID. These results suggest that DmMYB is required for the generation and/or maintenance of chromatin states that are permissive for the replacement of histone H3 by CID.

## G2-M (869-879)

869

**Cyclin A Regulates Mitotic Spindle Orientation**

H. Beamish, L. DeBoer, F. Stevens, B. G. Gabrielli; Centre for Immunology and Cancer Research, University of Queensland, Brisbane, Australia

Cell cycle progression is controlled by the ordered activation of the cyclin/cdk protein kinases. Progression into mitosis is controlled by the activation of cyclin B/cdk1, but progression through G2 phase and the events regulating the timing of cyclin B/cdk1 activation are still poorly understood. The role of cyclin A/cdk2 in cell cycle progression is complex. A pool is activated in early S phase and appears to be required for S phase progression, but a larger pool is activated in early G2 phase and its role is unknown. Inhibiting the cdk2 activity by over expressing a dominant negative cdk2 or using specific cdk2 inhibitors blocks progression into mitosis. We have investigated the function of cyclin A/cdk2 during G2 phase. We have established siRNA knockdown of cyclin A and its immediate upstream activator, cdc25B. Using time lapse videomicroscopy, fluorescence microscopy and flow cytometric techniques we have demonstrated the knockdown of either cyclin A or cdc25B produces a delayed entry into mitosis by blocking the activation of cyclin B/cdk1. However, the cells do eventually proceed into mitosis, where a number of condensed chromosomes fail to congress to the metaphase plate initiating the mitotic spindle checkpoint. The surprising feature of this arrest is that the established mitotic spindle with attached chromosomes rotates within the cell. Consequently there is a significant increase in the number of cells that undergo anaphase and cytokinesis out of the normal plane. The unexpected findings indicate a role for cyclin A/cdk associated with the mitotic spindle. To this end we have found cyclin A localised to the spindle poles prior to metaphase. This finding provides evidence for a previously unknown role for cyclin A/cdk in spindle formation and function in mitosis.

870

**A Potent G2-Phase Checkpoint Response to DNA Damage Induced by the Topoisomerase Inhibitor Adriamycin Requires Upregulation of Cyclin G2**

A. S. Arachchige Don, R. Dallapizza, C. Cowan, T. Weisenhorn, J. Duven, M. C. Horne; Pharmacology, University of Iowa, Iowa City, IA

Cyclin G2 (G2) is a unique, poorly understood stress-response protein possessing proliferation inhibitory activity. G2 expression is induced in response to DNA damage and other cellular stresses, but inhibited by activation of the proliferation promoting PI3K/AKT survival pathway. We showed that ectopic G2 expression results in a p53-dependent cell cycle arrest that also requires G2 association with protein phosphatase 2A, although the precise mechanism whereby elevated G2 promotes this arrest is unknown. We have recently determined that G2 is a centrosomal and nucleocytoplasmic shuttling protein. We found that treatment of Her2 positive breast cancer cells with Herceptin® (monoclonal antibody that blocks Her2 receptor-mediated PI3K/AKT pathway) or the PI3K Inhibitor (Ly294002), strongly upregulate cyclin G2 expression. *We hypothesize that dysregulation of G2 expression, interactions, or subcellular localization, leads to a loss of cell cycle control, ultimately promoting tumor formation.* Here, we seek to determine whether elevated G2 expression is necessary for the growth inhibitory effects induced by current chemotherapeutic agents relevant to cancer. Interestingly, Herceptin® treatment combined with a first line therapy that includes topoisomerase inhibitor and DNA damaging agent, Adriamycin®, improves survival rates and reduces the risk of breast cancer recurrence in premenopausal women. We treated cells with Adriamycin® in the presence or absence of cyclin G2 depletion via G2-specific shRNA and examined the effects on cellular proliferation via DNA flow cytometry. We found that Adriamycin® potently upregulates G2 expression and remarkably, depletion of endogenous cyclin G2 in Adriamycin®-treated cells abrogates the normal $G_2$-phase cell cycle arrest response. As chemotherapeutic agents induce G2 expression and G2 exhibits intrinsic growth inhibitory activity, understanding how G2 contributes to modulation of cellular proliferation is highly warranted and may provide future avenues for improved therapies.

871

**Synergistic Regulation of Mitosis by B Type Cyclins**

D. V. Soni, J. W. Jacobberger; Case Western Reserve University, Cleveland, OH

Cyclin B1/Cdk1 is a key regulator of mitosis with functions in nuclear membrane breakdown and the spindle checkpoint. We have measured the effect of siRNA-mediated depletion of cyclin B1 on the mitotic transition of HeLa cells by flow cytometry and time-lapse microscopy. The loss of cyclin B1 and its associated Cdk1 activity by at least 96% delayed the progression of cells from G2 through metaphase. Cyclin B1 depleted metaphase cells displayed a high frequency of lagging chromosomes that often failed to congress. However these cells entered anaphase and transited the rest of mitosis and cytokinesis with normal kinetics. In the presence of nocodazole, cyclin B1-depleted cells arrested in mitosis but eventually exited mitosis without completing cytokinesis and entered a 4C G1 state. Depletion of both cyclin B1 and cyclin B2 from HeLa cells by RNA interference resulted in a prolonged G2 arrest with failure to enter mitosis. We conclude that cyclin B1 is rate limiting for transition through metaphase and for maintaining the efficiency of chromosome congression and the spindle checkpoint. Moreover, while only one of the B type cyclins is required for the mitotic progression of HeLa cells, together cyclin B1 and B2 act synergistically to ensure efficient mitosis.

872

**TSPY Interacts with Cyclin B1 and Promotes G2/M Transition by Regulating the Cyclin B1/CDK1 Activity**

Y. LI, S. Oram, Y. C. Lau; Department of Medicine, University of California, San Francisco, VA medical Center, San Francisco, CA

The testis-specific protein Y-encoded (TSPY) gene has been mapped on the gonadoblastoma locus on the Y chromosome (GBY). TSPY is a tandemly repeated gene that expresses abundantly in normal testis, gonadoblastoma, testicular carcinoma-in-situ and seminoma, and in prostate cancer tissues and cell lines. TSPY contains a conserved domain present in a family of cyclin B binding proteins, including the oncoprotein SET. To confirm the putative cyclin B binding function, we have performed various interactive analyses between TSPY and cyclin B1, using the GST pull-down and co-immunoprecipitation assays. Two domains, residue #152-180 and #200-220 in the TSPY protein are important for its interaction with cyclin B1. A specific region, residue #302-314, in cyclin B1 is essential for binding to TSPY domains. Further, TSPY can be co-immunoprecipitated with a specific antibody against cyclin B1. TSPY colocalizes with cyclin B1 in both the nuclei and cytoplasm of transfected cells. It enhances the phosphorylation activity of cyclin B1/CDK1 complex on the substrate, histone H1. TSPY, in turn, can also be phosphorylated by the same complex. Over-expression of TSPY in HeLa cells shortens the G2/M transition. Our data suggest that TSPY regulates the cell cycle by binding to cyclin B1, thereby accelerating a rapid G2/M transition and modulating the cyclin B1/CDK1 activity.

873

**Tspy Expression Increases Cell Proliferation, Tumorigenesis, and Effects Cell Cycle Progression of Hela and Nih3t3 Cells**

S. W. Oram, X. Liu, Y. Lau; Medicine, UCSF- VA Medical Center, San Francisco, CA

TSPY is a gene located on the Y chromosome and has between 20-40 copies in humans. Deletion mapping of the gonadoblastoma locus on the Y chromosome has identified TSPY as the most likely candidate. Elevated levels of TSPY have been observed in gonadoblastoma specimens and TSPY expression has been observed in a variety of other tumor tissues: testicular germ cell tumors such as seminoma, non-seminoma, carcinoma *in situ*, and also in prostate cancer and melanoma. TSPY harbors a NAP/SET domain that is found in the oncoprotein SET and the nucleosome assembly protein 1 (NAP1), suggesting a possible role for TSPY in these processes. However, no cellular function has been definitely established for this Y-encoded protein. To determine if TSPY plays a role in cell cycle regulation and/or oncogenesis, we have established a Tet-off regulated system for TSPY expression in HeLa and NIH3T3 cells. We demonstrate that expression of TSPY increases cell proliferation *in vitro* and tumorigenicity *in vivo*. TSPY mediates changes in the cell cycle profile of these cells, resulting in a smaller population of cells in the G2/M phase of the cell cycle. Using cell synchronization techniques, we show that TSPY appears to effect a G2/M transition at a faster rate. These findings provide the first insights on the role of TSPY, demonstrating that TSPY is involved in the regulation of cell cycle progression, and abnormal expression of TSPY increases cell proliferation and tumorigenicity of host cells in culture and nude mice.

874

**Cantharidin-Induced Mitotic Arrest and Subsequent Apoptosis is Associated with the Formation of Aberrant Mitotic Spindles and Improperly Aligned Chromosomes Resulting, in Part, from the Suppression of PP2Aα Activity in Human Cells**

K. M. Bonness,[1] B. Rutland,[2] N. Dean,[3] R. Honkanen[2]; [1]Center for Lung Biology, Dept of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL, [2]Dept of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL, [3]ISIS Pharmaceuticals, Carlsbad, CA

Cantharidin is an inhibitor of serine/threonine protein phosphatases (PP1, PP2A, PP4, and PP5) that displays marked anti-tumor activity. To explore the molecular mechanisms associated with the anti-tumor activity of cantharidin, we examined the cellular effects produced in human cells. Cantharidin produces an apoptotic response at concentrations inhibiting >50% of the activity in the serine/threonine phosphatases. Additionally, cantharidin produces a dose-dependent growth arrest during G2/M-phase. The maximal response is achieved at 3-4μM cantharidin, which is sufficient to completely suppress the activity of PP2A, PP4 and PP5 while partially suppressing the activity of PP1. FACS analysis of synchronized A549 cells treated with cantharidin revealed a typical progression through the cell cycle until G2/M-phase where they arrested. The addition of cantharidin to synchronized cells at timed intervals, which were consistent with the G1-, S-, and G2-phases of the cell cycle, also triggered G2/M growth arrest. This suggests that the critical event arresting cell cycle progression occurs during the G2/M-phase. Immunostaining indicates that growth-arrest occurs prior to the completion of mitosis since it is associated with the formation of numerous abnormal mitotic spindles and condensed chromosomes that fail to align properly along the metaphase plate. Prolonged disruption of both the mitotic spindle and chromosome alignment is followed by an apoptotic response that likely contributes to the antitumor activity of cantharidin. Furthermore, inhibition of phosphatase expression using siRNA and antisenses oligonucleotides indicates that PP2Aα is crucial for completion of mitosis and progression through the cell cycle. Fluorescent microscopy of PP2Aα anti-sense treated cells illustrates a similar phenotype in which chromosomes fail to align properly during a prolonged growth arrest and subsequent apoptosis.

875

**ERK1/2 and Mitosis in Mammalian Cells**

M. Shinohara,[1,2] A. V. Mikhailov,[2] C. L. Rieder[1,2]; [1]Department of Biomedical Sciences, State University of New York, Albany, NY, [2]Lab. of Cell Regulation, Department of Molecular Medicine, Wadsworth Center, Albany, NY

The extracellular signal-regulated kinase (ERK) 1/2 pathway is required for the $G_1$/S transition. When activated it facilitates protein syntheses, induces cytoskeletal rearrangement and activates transcription of cell cycle regulators including cyclins D and E. After S phase, however, the role of ERK signaling becomes controversial. Biochemical studies suggest that ERK1/2 activity is required for the $G_2$/M transition, Golgi fragmentation and the spindle assembly checkpoint. Yet evidence is accumulating that receptor-mediated ERK1/2 activation is suppressed during mitosis. The goal of our study was to determine if ERK activity is required for mammalian cells to progress normally into and through mitosis. For this we used high-resolution time-lapse microscopy to follow cell cycle progression both temporally and phenotypically in human retinal pigment epithelia (hTERT-RPE1) and rat kangaroo ($PtK_1$) cells treated with either a small molecule inhibitor (U0126) or activator (TPA) of the ERK pathway. To avoid the effects of ERK pathway intervention on gene expression we treated cells during late $G_2$ (antephase), approx. 1 hr before they became committed to mitosis. We found that these treatments had no influence on the kinetics of mitotic entry and exit in both cell lines. Treated cells also exhibited normal chromosome condensation, nuclear envelope breakdown, chromosome congression, sister chromatid separation and cytokinesis. Immunofluorescence revealed normal, bipolar spindle formation. We also found that ERK1/2 activity was dispensable for initiating or maintaining the spindle assembly checkpoint. There was, however, a slight increase in the early $G_2$ cell population 1-4 hrs after U0126 treatment. Together, the ERK1/2 cascade is not required for late $G_2$ cells to enter or proceed through mitosis.

876

**The Presence/Activity of Chk1 in the UV-induced Cellular Response in the Purple Sea Urchin, *Strongylocentrotus purpuratus***

G. A. Goschke, N. L. Adams; Biological Sciences, California Polytechnic State University, San Luis Obispo, CA

Chk1 is a protein kinase known in some organisms to be involved in ultraviolet radiation (UVR)-induced DNA damage response and may regulate proteins downstream involved in the G2/M transition of the cell cycle. G2/M arrest is regulated by the mitosis-promoting factor (MPF), which is a complex of Cdc2 and cyclins. Cdc2 is activated by Cdc25 (a protein phosphatase), a crucial step in the entry into M-phase of the cell cycle. Phosphorylation of Cdc25 at ser 216 prevents activation of Cdc2. Cdc25 is phosphorylated throughout interphase, but not in mitosis. Chk1 (an upstream kinase) in turn phosphorylates Cdc25 and thereby inhibits mitosis. Previous studies of sea urchin embryos have shown that UVR exposure induces cellular response to UVR damage via cell cycle delays at the G2/M transition, but the molecular aspects of this response are unclear. In addition to this delay, when exposed to UVB (280-320 nm), sea urchin gametes and zygotes exhibit both immediate and delayed effects on both morphology and physiology in mitosis in response to UVR. Our previous results demonstrated that UV-irradiation of sea urchin eggs causes a delay in dephosphorylation of Cdc2 at tyr 15 (preventing activity of Cdc2). In our current study, we have examined whether an upstream regulator of Cdc2 activity, Chk1, is present in sea urchin eggs and embryos and has a similar function in response to UVR as exists in other organisms. We performed sequence alignments with human Chk1 and the currently available sea urchin genome sequence. We performed SDS PAGE and western blotting of sea urchin embryo lysates using antibodies generated against human Chk1. Our results indicate that Chk1 is likely to be present in sea urchins eggs and embryos.

877

**Non-catalytic Function for Xenopus ATR in the S/M Checkpoint Response**

T. D. McSherry,[1] A. A. Kitazono,[2] A. Javaheri,[1] S. J. Kron,[1] P. R. Mueller[3]; [1]Center for Molecular Oncology, University of Chicago, Chicago, IL, [2]Biochemistry, Cellular, and Molecular Biology, University of Tennessee at Knoxville, Knoxville, TN, [3]Biology, University of Texas at San Antonio, San Antonio, TX

The ATR family of checkpoint kinases is essential for an appropriate checkpoint response. These large kinases phosphorylate and modify multiple cell cycle and checkpoint factors, leading to cell cycle arrest, DNA repair, and induction of apoptosis. The catalytic domain of all ATR family members comprises only a small fraction of the total protein, leaving unresolved the function of the majority of the protein. Biochemical structure/function analyses of Xenopus ATR (XATR) in Xenopus extracts have been hampered by the large size of ATR. To address this problem, we have used a cross species approach designed to take advantage of the high degree of homology between metazoan and yeast ATR family members. We examined XATR function by exploring its ability to suppress the S/M checkpoint deficient phenotype of a *S. cerevisiae* ATR homolog *mec1* mutant. Expression of wild type, kinase dead, or an amino terminal portion of XATR in a *mec1* mutant suppresses the S/M checkpoint defect and induces the DNA damage dependent mitotic cell cycle arrest. This suppression functions independently of Rad9 modification and Rad53 activation. In addition, XATR expression suppresses the checkpoint defects of other various checkpoint mutants. Together,

these results indicate that XATR is not functioning through the established S/M checkpoint pathways. Instead, we find that XATR suppression of the *mec1* mutant checkpoint defect requires Mad1/2 of the spindle assembly checkpoint, suggesting that XATR is functioning through this pathway. Finally, expression of kinase dead and truncation constructs of the yeast ATR homolog, MEC1, in the *mec1* mutant also partially suppresses the S/M checkpoint defect and induces a mitotic cell cycle arrest in a Mad1 dependent manner. Thus, the link between the non-catalytic region of the ATR kinase family and the spindle checkpoint pathway is conserved between yeast and metazoans.

878

### Control of Mitotic Entry by MIH1, the Budding Yeast Homolog of CDC25

G. Pal, D. R. Kellogg; Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Cruz, Santa Cruz, CA

Entry into mitosis is induced by activation of cyclin-dependent kinase 1 (Cdk1) and is highly regulated. The Wee1 kinase delays entry into mitosis by phosphorylating and inhibiting Cdk1, while the Cdc25 phosphatase promotes entry into mitosis by dephosphorylating Cdk1. In fission yeast, Wee1 and Cdc25 are thought to coordinate cell growth with entry into mitosis; however, the physiological signals and molecular mechanisms that regulate Wee1 and Cdc25 are poorly understood. Recent work has shown that the budding yeast homolog of Wee1 may also function to coordinate cell growth with entry into mitosis, but the functions of budding yeast Cdc25 have not been carefully characterized. The homolog of Cdc25 in budding yeast is called Mih1. We found that loss of Mih1 causes cells to delay at G2/M while still undergoing growth, indicating that the basic functions of fission yeast Cdc25 have been conserved in budding yeast Mih1. To better understand the Mih1-dependent mechanisms that control entry into mitosis, we are characterizing how Mih1 is regulated. Interestingly, Mih1 undergoes constitutive hyperphosphorylation during the cell cycle and becomes dephosphorylated as cells enter mitosis. Dephosphorylation of Mih1 is triggered by mitotic Cdk1 activity, which suggests that Cdk1 activates a phosphatase that dephosphorylates and further activates Mih1. We are currently working to identify the phosphatase and the kinase that regulate Mih1.

879

### Dot2, the Fission Yeast Homolog of Human EAP30, Blocks Meiotic Spindle Pole Body Amplification

Y. Jin, J. J. Mancuso, S. Uzawa, D. Cronembold, W. Z. Cande; Department of Molecular and Cell Biology, UC, Berkeley, Berkeley, CA

Centrosome aberrations caused by misregulated centrosome maturation result in defective spindle and genomic instability. Here we report that the fission yeast homolog of human EAP30, Dot2, negatively regulates meiotic Spindle Pole Body (SPB, the yeast equivalent of centrosome) maturation. dot2 mutants show excess electron-dense material accumulating nearby SPBs seen as enlarged SPBs or multiple SPB-like structures, which we refer to as Aberrant Microtubule Organization Centers (AMtOCs). SPB aberrations were associated with elevated Pcp1 protein level, the fission yeast ortholog of pericentrin/kentrin. Replacement of the endogenous pcp1+ promoter by a foreign promoter can significantly suppress AMtOCs in dot2-439 cells, indicating that Dot2 regulates pcp1+ expression. Dot2::GFP is colocalized with Mei2 as a spot on a chromosome in the nucleus and genetically interacts with Sme2, a RNA that is binds to Mei2. Our findings provide the first evidence that a member of the widely conserved EAP30 family is required for maintenance of genome stability by regulating SPB maturation and also indicate a meiosis-specific regulation of SPB maturation. In addition to its nuclear function, Dot2 is also found involved in vacuolar protein transport and sorting in vegetativly- growing fission yeast cells (Kaoru Takegawa, personal communication).

## Cytokinesis I (880-897)

880

### Understanding cell division: A forward genetics approach

J. C. Canman, B. Bowerman; Institute of Molecular Biology, University of Oregon, Eugene, OR

Accurate cell division is crucial for preventing aneuploidy during both development and cell differentiation. The early *C. elegans* embryo is a powerful model system for identifying genes required for cell division. Many genes required for cell division in *C. elegans*, however, are also required for germline development and gonad function. Therefore disruption of these genes results in sterility- precluding analysis of any potential gene function in the early embryo. To overcome this pitfall, we have developed a forward genetics approach to isolate conditional alleles of genes involved both in gonad function as well as cell division in the early embryo. We first isolated ~100 mutants that could make viable offspring at the permissive temperature (15°C) but became sterile if shifted to the restrictive temperature (26°C) at the L4 larval stage (after the majority of gonad development has occurred). We then took advantage of the conditional nature of these mutations to determine which mutants are defective in cell division. To do this, we shifted adult worms from the permissive to the restrictive temperature after they began producing embryos, and then imaged the first two embryonic cell cycles using DIC microscopy. Using this approach, we have screened through about half of the mutant collection and have identified four several mutants with both sterility defects and embryonic cell division defects. We are currently examining the cellular defects in these mutants, and mapping the mutations to determine gene identity.

881

### Rho-kinase Plays a Pivotal Role in Triggering the Earliest Shape Changes of Cytokinesis in *Drosophila* S2 Cells

G. R. X. Hickson,[1] A. Echard,[2] P. H. O'Farrell[1]; [1]Dept. Biochemistry & Biophysics, University of California, San Francisco, San Francisco, CA, [2]Cnrs umr144, Institut Curie, Paris, France

Animal cell cytokinesis is characterized by a sequence of dramatic cortical rearrangements. The mechanisms that coordinate these rearrangements and couple them with mitosis are largely unknown. In efforts to explore the initiation of cytokinesis, we have focused on anaphase cell elongation: the earliest cell shape change that occurs in concert with anaphase B, prior to cytokinetic furrowing. Using RNAi and live video-microscopy in *Drosophila* S2 cells, we implicate Rho-kinase (Rok) and myosin II in anaphase cell elongation. *rok* RNAi prevented equatorial myosin II recruitment, prevented cell elongation and caused a remarkable spindle defect where the spindle poles collided with an unyielding cell cortex and the interpolar microtubules continued to extend and buckled outwards. Disruption of the actin cytoskeleton with Latrunculin A alleviated the spindle defect, consistent with cortical dysfunction being primary. Despite failure of cell elongation and spindle disruption, cytokinesis often succeeded following *rok* RNAi. Co-suppression of the myosin binding subunit (MBS) of myosin phosphatase, which is known to antagonize Rok

activation of myosin II, suppressed any failures in cytokinesis that were induced by *rok* RNAi, but did not suppress the failures in cell elongation. The marked sensitivity of cell elongation to Rok depletion was highlighted by RNAi to other genes in the Rho pathway, such as *Pebble*, *RacGAP50C* and *Diaphanous* which had little or no effect on cell elongation but had profound effects on furrowing. Our results show that cell elongation in anaphase requires Rok and myosin II dependent cortical rearrangements and the marked sensitivity of cell elongation to depletion of these gene products as opposed to other regulators of cytokinetic furrowing suggests regulatory distinctions.

882

**RCK-1, the Mammalian RACK1 Homolog, is Required for Cytokinesis in *C elegans* Embryos**

E. Ai, A. R. Skop; Department of Genetics and Medical Genetics, University of Wisconsin - Madison, Madison, WI

Cytokinesis is an essential event required to partition chromosomes, organelles and cytoplasm to daughter cells. In animal cells, it is driven by a cortical actin-myosin contraction that forms the cleavage furrow and pinches the cell into two. The mechanisms necessary for cleavage furrow establishment and completion remain unknown. We identified RACK1 (Receptor for Activated C Kinase 1) in a proteomic screen of isolated midbodies and showed that the *C. elegans* homolog, RCK-1/K04D7.1, was required for cytokinesis (Skop et al., 2004). Because RACK1 is thought to be a scaffolding protein that mediates interactions among many signaling molecules, the requirement for this protein suggests the necessity of regulatory cascades in furrow establishment and/or completion. To address these possibilities, we are using *in vivo* imaging techniques combined with genetic and biochemical assays in both *C. elegans* and mammalian cells to further characterize the role of RCK-1 in cytokinesis. In *C. elegans* embryos, RCK-1 depletion by fRNAi results in early (38%) and late (11%) cytokinesis failures and displays 58% embryonic lethality. Defects in meiotic cytokinesis are also observed in which polar bodies fail to extrude. The spindle midzone is properly established in early anaphase, yet falls apart in late anaphase. In addition, chromosomes fail to align into the metaphase plate (10/12 embryos) and lagging chromosomes (9/12 embryos) and chromatin bridges (3/12 embryos) are seen. Furthermore, cell cycle delays are observed only during pronuclei migration and between the first and second divisions. These findings suggest that RCK-1 is required for multiple events and possibly integrates a variety of regulatory pathways during cytokinesis. Skop, A. R., Liu, H., Yates, J. 3rd, Meyer, B. J., Heald, R. *Science* 305, 61 (2004).

883

**Regulation of Cytokinesis by a Novel GAP Protein**

A. V. Binda,[1] A. Gromley,[2] S. J. Doxsey[1]; [1]Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, [2]St. Jude's Children's Research Hospital, Memphis, TN

Completion of cell division is marked by the physical separation of two daughter cells in a process known as cytokinesis. Previously, we identified centriolin as a protein necessary for the final stage of cytokinesis, abscission. Centriolin localizes to the maternal centriole and to a ring structure at the midbody of telophase cells. Depletion of centriolin results in cytokinesis defects, most notably persistent intracellular bridges that remain between daughter cells. To determine how centriolin functions in cytokinesis, we performed a yeast two-hybrid screen using a centriolin domain homologous the *S. cerevisiae* protein, Nud1p. We identified, cloned, and sequenced a previously uncharacterized cDNA encoding a protein with homology to GTPase activating proteins (GAPs). Homology searches revealed a coiled-coil domain within the C-terminus of the GAP and a putative C2 domain within the N-terminal region. To verify the interaction of centriolin and the GAP, we performed coimmunoprecipitation assays and demonstrated an interaction between endogenous centriolin and the GAP domain. An antibody raised against the GAP stained the midbody of telophase cells, as well as at the centrosome and *trans*-Golgi network (golgin 97) throughout the cell cycle. Midbody localization was confirmed by ectopic expression of GFP-tagged constructs encoding that GAP domain and the C-terminal domain of the GAP. Midbody localization of the GAP appears to be coincident with the endosomal proteins, Rab11 and FIP3, suggesting a role for the GAP in vesicle delivery to the midbody during abscission. Consistent with this hypothesis, we found that overexpression and siRNA-mediated depletion of the GAP result in cytokinesis defects in ~40% of cells. We are currently working to identify the GTPase target of the GAP and its mechanism of action during cytokinesis.

884

**Equatorial Accumulation of RhoA-GTP During Cytokinesis**

A. J. Piekny, M. Glotzer; Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

RhoA GTPase is essential for cytokinesis in many eukaryotes as determined by genetic or biochemical studies. During anaphase the generation of the active form of RhoA, RhoA-GTP, requires both the RhoGEF ECT2 and the RhoGAP CYK-4. CYK-4, part of the centralspindlin complex, binds ECT2 and these two proteins colocalize on the central spindle. CYK-4 may act by relieving the autoinhibition of ECT2. RhoA-GTP activates several downstream effectors required for furrow formation, ingression and stabilization. To follow the localization of RhoA during cytokinesis, we generated a probe consisting of YFP fused to full-length *C. elegans* RhoA (YFP:RhoA). Unlike YFP:human RhoA, the probe based on the *C. elegans* protein localizes discretely to the equatorial cell cortex. In addition, the cortical localization of this probe is enhanced by overexpression of a RhoGEF domain and reduced by overexpression of a RhoGAP domain. Finally, YFP:RhoA localizes similarly to a RhoA-GTP binding domain (RBD) probe in sea urchin embryos and likely represents the RhoA-GTP pool. Disruption of the central spindle by MKLP1 depletion results in variable expansion of the RhoA-GTP domain. Thus, the central spindle contributes to the localization of active RhoA but a redundant mechanism contributes to RhoA-GTP localization. The differential localization of ceRhoA and hsRhoA can be attributed to a few amino acid changes, perhaps by altering the affinity toward specific binding factors. ceRhoA and hsRhoA are 94% identical in their NH2 terminus, but diverge in the C-terminus. The generation of chimeric constructs verified that the differences in their localization patterns are likely due to amino acid changes in the C-terminal portion of the protein, distinct from the CAAX box. Furthermore, we could separate regions required for RhoA-GTP accumulation and cortical localization. Experiments are in progress to identify proteins that specifically bind RhoA during its accumulation in anaphase.

885

**Dissecting the Role of Rho-mediated Signaling in Contractile Ring Formation**

K. Kamijo,[1] N. Ohara,[1] M. Abe,[1] T. Uchimura,[2] H. Hosoya,[2] J. Lee,[1] T. Miki[1]; [1]Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, MD, [2]Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Japan

Proper positioning of the cleavage plane ensures chromosome separation and asymmetric cell division-critical steps for genome stability and

development, respectively. Although microtubules are known to provide a specification signal for positioning of the contractile ring during cytokinesis, the nature of the signal remains unknown. The implication of the small GTPase Rho in cytokinesis has been reported but it remains unclear whether Rho serves as the specification signal for positioning of the contractile ring. We have found that Rho translocates to the equatorial cell cortex before furrow ingression. The Rho specific inhibitor C3 exoenzyme prevented this translocation and disrupted contractile ring formation. Furthermore, RhoGEF ECT2 RNAi prevented translocation of Rho to the equatorial cell cortex and inhibited contractile ring formation. These results indicate that active Rho is required for contractile ring formation and that ECT2 regulates activation of Rho and its translocation to the equatorial cell cortex. MgcRacGAP and MKLP1 kinesin are required for assembly of the central spindle. We found that ECT2 co-localized with MgcRacGAP and MKLP1 at the central spindle and that the localization of ECT2 at the central spindle was dependent on MgcRacGAP and MKLP1. In addition, we have developed the experimental furrow induction system, using the Cdk1 inhibitor Roscovitine. We found that Roscovitine promoted premature exit from mitosis and induced ectopic furrows. Using this system, we have found that furrow formation depends on bundled microtubules and that Rho accumulates at the ectopic furrows. Although microtubules were bundled, ECT2 RNAi inhibited Roscovitine-induced ectopic furrowing and accumulation of Rho. Thus, we conclude that the Rho-mediated signaling acts as a link between the central spindle and contractile ring formation.

886

**Polo-like Kinase Regulates the Cortical Cytoskeleton via Multiple Rho-GTPase Regulators**

S. Yoshida,[1] K. Kono,[2] S. Bartolini,[1] D. Lowery,[3] M. Yaffe,[3] Y. Ohya,[2] D. Pellman[1]; [1]Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, [2]University of Tokyo, Kashiwa, Japan, [3]Massachusetts Institute of Technology, Boston, MA

Cytokinesis is a dynamic process that involves highly coordinated regulation of the cortical cytoskeleton and cell cycle machinery. Cytokinetic actin ring is assembled before mitotic exit but how the cell cycle signal regulates cortical cytoskeleton reorganization during anaphase was not understood very well. We found that the Polo-like kinase Cdc5 mutants are defective in cytokinesis and in the reorganization of cortical actin cytoskeleton due to mislocalization of the cell polarity factors. A functional genomic screen was performed to identify candidate new Cdc5 substrates. By this method, Rho guanine nucleotide exchange factors (RhoGEFs) and Rho GTPase-activating proteins (RhoGAPs) were identified. Our biochemical analysis suggests that these Rho-regulators are bona fide Cdc5-substrates and these interactions were mediated by Cdk-dependent phosphorylation of the Cdc5 targets. Genetic analysis of the gain and loss of function mutations further validated an important role of Cdc5 in the regulation of Rho-GTPases. Our findings thus reveal a novel function for polo: regulation of Rho-GTPases and the cytoskeleton. Regulation of multiple Rho regulators by Polo provides a mechanism that co-ordinates cortical cytoskeletal regulation and mitotic exit to ensure successful cell division.

887

**De Novo Self-assembly of the Actomyosin Contractile Ring in Anucleate Cells**

P. T. Tran,[1] Y. Huang,[2] S. Oliferenko,[2] M. K. Balasubramanian[2]; [1]Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA, [2]Cell Division Group, Temasek Life Sciences Laboratory, Singapore, Singapore

The actomyosin contractile ring, a multi-protein structure that contains F-actin, type II myosin and several other proteins, is important for cytokinesis in unicellular and multicellular eukaryotes. In animal cells, this ring is assembled in the plane perpendicular to that of the mitotic spindle long axis upon onset of anaphase. Depending on the cell type, the signals are postulated to come from the spindle midzone containing antiparallel microtubules, the centrosome-organized astral microtubules which interact with the cell cortex, and/or the metaphase chromosomes which contain signaling passenger proteins. Here, we present surprising evidence for the de novo self-assembly of actomyosin rings in anucleate fission yeast cells. Our findings suggest that mechanisms triggering self-assembly of the actomyosin contractile ring can occur independently of components such as spindle midzone, astral microtubules, centrosomes or chromosomes. * P.T. Tran and Y. Huang contributed equally to this work.

888

**Tracking Actin Dynamics with Quantum Dots during Contractile Ring Assembly**

W. Chen, D. Zhang; Zoology/CGRB, Oregon State University, Corvallis, OR

The contractile ring assembles during late anaphase and remains highly dynamic as it constricts the cell during cytokinesis. Although both newly-polymerized actin filaments and pre-existing actin cables may contribute to the ring dynamics, it is unclear whether these actin filaments are static or dynamic as they assemble into the ring. To address this question, we co-labeled actin filaments in insect spermatocytes by microinjection with trace amounts of Alexa 488 phalloidin and Qdot 655 phalloidin conjugates. As Qdot 655 phalloidin intermittently decorates actin filaments, it forms quantum dot speckles along the length of Alexa 488 phalloidin-stained actin filaments. Being highly-resistant to photobleaching, these bright Qdot speckles provide precise reference points for analyses of shortening or elongation of an actin filament and its movement relative to other filaments or cellular constituents during cytokinesis. Our results show that during late anaphase and early telophase, stable actin filaments move towards the equatorial cortex, aligning into V-shaped cortical structures around the cell equator. As cytokinesis proceeds, the V-shaped actin filaments merge into a belt of parallel actin bundles perpendicular to the spindle axis. It appears that *de novo* assembly of actin filaments also contributes to the enrichment of actin filaments in the contractile ring, as some speckles incorporate into the ring independent of preexisting actin filaments. This approach may permit the tracking of actin dynamics during other cellular events and in other cell types which involve studying the assembly and contraction of the actomyosin based contractile system.

889

**Actin Cross-linking Protein α-actinin Regulates Cell Cleavage during Cytokinesis in Tissue Cultured Cells**

S. A. Mukhina,[1] Y. Wang,[2] M. Murata-Hori[1]; [1]Temasek Life Sciences Laboratory, Singapore, Singapore, [2]University of Massacusetts Medical School, Worcester, MA

Cytokinesis is a spatially and temporally regulated event crucial for cell proliferation. Despite much attention, it is still unclear how cytokinesis is regulated. Due to the existence of strong cortical forces and the concentration of actin filaments and myosin II along the equator, it is principally

believed that an equatorial contractile ring generates forces to produce two daughter cells. However, the regulation of cell cleavage appears to be more complex, and may involve modulation of actin filament structures. Thus, to understand the detailed mechanism of the cell cleavage, it is important to identify the functions of actin modulating proteins at a high spatial and temporal resolution. In this study, we analyzed the detailed functions of an actin cross-linking protein α-actinin during cytokinesis in tissue cultured cells. Using GFP technology, we found that α-actinin accumulated along the equator underwent gradual dissipation as cytokinesis progressed. Overexpression of α-actinin induced excessive bundling of actin filaments in the equatorial region, which frequently led to cytokinesis failure. To the contrary, depletion of α-actinin by siRNA promoted collapse of the equatorial cortex and caused accelerated cell cleavage. In order to investigate if depletion of α-actinin along the cleavage furrow facilitated cytokinesis, we performed local inactivation of α-actinin at one side of the cleavage furrow by chromophore assisted laser inactivation. Strikingly, the laser-irradiated side of the equator developed a deep furrow while irradiation of GFP alone did not affect cytokinesis, supporting the idea that inactivation of α-actinin at the equator facilitates cytokinesis. Our results strongly suggest that cytokinesis involves the regulation of actin filaments cross-linking by α-actinin at the equator.

890

**Actin Crosslinking and Severing Controls Cytokinesis Contractility Dynamics and Intercellular Bridge Separation**

E. M. Reichl, D. N. Robinson; Cell Biology, Johns Hopkins University, Baltimore, MD

Cytokinesis is the mechanical separation of a cell into two daughter cells. A genetic screen to isolate factors that inhibited cytokinesis was carried out by using a cDNA overexpression library in *Dictyostelium discoideum.* An actin isoform, the actin polymerization regulator VASP, and the actin crosslinkers p34 and fimbrin were identified in the screen. Because the fimbrin overexpression phenotype was highly penetrant, we chose to investigate it in more detail and to contrast it with the actin filament severing protein cofilin. Both fimbrin and cofilin are required for wild type bridge morphology and cytokinesis efficiency. Interestingly, the mutant morphology of cells with increased fimbrin levels differed, depending on whether myosin II was present, and the phenotype of the *myosin II*: *fimbrin OE* cytokinesis may be best explained by considering the fluid dynamics of cytokinesis. In summary, the cell appears to finely regulate the mechanics of the actin cortex to achieve efficient, stereotypical cytokinesis.

891

**Testing the Role of Astral Microtubules in Contractile Ring Assembly**

L. I. Strickland, K. Johanson, D. R. Burgess; Biology, Boston College, Chestnut Hill, MA

Astral microtubules (MTs) communicate with the cell cortex during anaphase in echinoderm eggs to stimulate the assembly of the contractile ring for cytokinesis. The polar relaxation model asserts that astral MTs relieve tension at the polar cortex, resulting in contraction at the equator where tension remains high. The equatorial stimulation model proposes that MTs emanating toward the equator promote contractility of the equatorial cortex. We report a series of experiments to determine whether astral MTs exert a positive or negative influence on the contractility of the cortex in dividing sea urchin eggs. We used local application of nocodazole (NZ) to locally depolymerize astral MTs at one pole or the equator at the time of MT-cortical contact. We used a GFP-tagged F-actin binding domain of the eutrophin protein to visualize the contractile ring. When NZ was applied locally with a micropipette to one pole, ring assembly was displaced toward the opposite pole by an average of 5 um. NZ released the polar astral MTs from their attachment to the polar cortex at the site of application. However, the position of the mitotic apparatus relative to the opposite pole was not affected by NZ application so that the displacement of the contractile ring resulted in a cleavage furrow that initiated off center relative to segregating chromosomes. As cytokinesis progressed, the contractile ring centered by sliding across the cell toward the NZ treated pole, resulting in two daughter cells of equal volume. When NZ was applied to the equatorial cortex on one side, furrowing was delayed or blocked at the site of application but not at the opposite side resulting in a unilateral furrow. These results support the equatorial stimulation model and are not consistent with the polar relaxation model for cytokinesis.

892

**Microtubules Regulate Organization and Dynamics of Actin during Cytokinesis**

K. T. Murthy, C. J. Fagerstrom, P. Wadsworth; Biology Department, University of Massachusetts, Amherst, Amherst, MA

During cytokinesis, an F-actin and myosin II containing contractile ring assembles at the equatorial cortex and functions to divide the cell into two daughter cells. Microtubules of the mitotic spindle specify the site of contractile ring formation, but the mechanism by which this is accomplished is not known. To gain insight into how microtubules contribute to actin organization and dynamics during cytokinesis, we imaged GFP-actin in control cells and in cells treated with 33uM nocodazole to diassemble microtubules. Cells that were treated with nocodozole immediately after anaphase onset were blocked in cytokinesis with no distinct actin accumulation at the equatorial cortex. When cells were treated with nocodazole approximately one minute after anaphase onset, cytokinesis continued, although more slowly than control cells. In these cells, the contractile ring was dramatically increased in width and actin was observed to move toward and accumulate at the equatorial region. Under these conditions the microtubules were rapidly disassembled, although a subset of central spindle microtubules were differentially stable. In all nocodazole treated cells, GFP-actin organization was perturbed; actin was observed to accumulate in non-equatorial regions of the cortex and in some cases wave-like cortical contractions were observed. Active myosin light chain, identified by staining with antibody to phosphorylated myosin light chain, was observed at the contractile ring in control cells, but was missing in nocodozole treated cells. FRAP experiments in nocodozole treated cells demonstrate that actin remained highly dynamic in cells that continued to cleave, but turnover was suppressed in cells that were blocked in cytokinesis. Our results demonstrate that microtubules direct actin flow towards the equator during cytokinesis and prevent actin accumulation in other regions of the cortex. In addition, microtubules dictate the width of the contractile ring.

893

**Cdc42 Stabilizes Microtubules Along the Equatorial Cortex During Cytokinesis**

X. Zhu,[1] C. Foong,[1] S. Ahmed,[2] M. Murata-Hori[1]; [1]Mammalian Cell Biology, Temasek Life Sciences Laboratory, Singapore, Singapore, [2]Neural Stem Cell Laboratory, Institute of Bioengineering, National University of Singapore, Singapore, Singapore

Cytokinesis is a fundamental process essential for cell proliferation. In animal cells, the anaphase spindle has been implicated in the stimulation of

cytokinesis. Recent analyses of microtubules and microtubule binding proteins in the anaphase spindle suggest that the stabilized microtubules along the equator are involved in the regulation of cytokinesis. However, how the microtubules along the equator are stabilized and how the stabilized microtubules stimulate cytokinesis remain unanswered. An attractive candidate molecule regulating these processes is the small GTPase Cdc42. Since Cdc42 is involved in cytokinesis and in the regulation of microtubules in cell migration and polarity, we speculated that Cdc42 might regulate microtubules during cytokinesis. To address this hypothesis, we constructed TAT fused dominant active and negative mutants of Cdc42 and applied them to anaphase NRK cells. Interestingly, we found that the entire cortex appeared to be activated during cytokinesis in the cells treated with dominant active and negative mutants of Cdc42, which frequently led to cytokinesis failure. To analyze the dynamics of microtubules in these cells, we applied TAT fused dominant active and negative mutants of Cdc42 to the anaphase NRK cells expressing GFP-tubulin and took time-lapse imaging of microtubules. Kymographic analyses revealed that the astral microtubules became stabilized in the cells treated with dominant active and negative mutants of Cdc42, while dynamic astral microtubules were observed in the control cells. 3D reconstruction revealed the abnormal organization of midzone microtubules in cells treated with dominant active and negative mutants of Cdc42. We also found that dominant active and negative mutants of Cdc42 predominantly accumulated along the equatorial cortex during cytokinesis. Taken together, we suggest that Cdc42 is involved in the stabilization of microtubules along the equator, which may in turn stimulate cytokinesis.

894

### Mechanical Feedback Regulation of Cytokinesis

J. C. Effler,[1] S. Kee,[2] M. Tran,[3] P. A. Iglesias,[1] D. N. Robinson[4]; [1]Electrical and Computer Engineering, Johns Hopkins University, Baltimore, MD, [2]Luther College, Decorah, IA, [3]Elmira College, Elmira, NY, [4]Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

Cytokinesis, the physical separation of a mother cell into two daughter cells, is mechanically regulated by various actin-crosslinking and actin-based motor proteins which differentially localize to the polar or cleavage furrow cortex. How cells regulate and control their progression through the various shape changes of cytokinesis in a variety of mechanical environments is not well understood. We postulate that mechanical feedback mechanisms exist which help to govern this process. To test this hypothesis, we apply physiologically relevant mechanical loads to dividing cells at differing stages of cytokinesis and monitor the distribution of fluorescently tagged proteins. Cells in anaphase respond to these loads by recruiting myosin-II and cortexillin to the portion of the cortex where the load is applied. This redistribution allows the cell to escape the pipette. Thereafter, myosin-II and cortexillin are recruited to the cleavage furrow cortex. Neither protein redistributes under equivalent mechanical loads in interphase cells. Through these experiments we have begun to uncover how cells transduce mechanical signals to regulate cellular shape.

895

### Probing Cytoplasmic Structures and Forces in Animal Cells during Cytokinesis

T. Iwasaki, Y. Wang; University of Massachusetts Medical School, Worcester, MA

Biochemical, cell biological, and genetical studies have provided clues on many aspects of the mechanism of cytokinesis. However, little is known about mechanical environment of the cytoplasm in dividing cells beyond what was demonstrated decades ago using flexible microneedles. To detect the distribution of forces, we injected high molecular weight, linear polyacrylamide (PAA) into NRK cells and monitored their shape and movements. PAA in interphase cells appeared as scattered, tangled aggregates with irregular shapes. However, when the cell entered prometaphase, PAA aggregates coalesced into a small number of spheres as if there were an increase in the structure of the surrounding cytoplasm, possibly due to an increase in the density, stability, or rigidity of the cytoskeleton that excludes PAA. In contrast, PAA in interphase cells rounded up by trypsin remained as scattered aggregates. Following anaphase onset, PAA aggregates near the equatorial region moved rapidly into the spindle midzone. The pattern of movements, and the insensitivity to cytochalasins, suggested that the movement involved localized weakening of cytoplasmic structures in the interzone rather than pushing forces of the ingressing cortex. Aggregates outside the equatorial region showed no consistent movements during anaphase. These results suggest that, in addition to cortical forces, changes in cytoplasmic structures may play an important role in the shape change, midzone formation, and cleavage process of dividing cells.

896

### Mob1 Associated Proteins and their Roles in Regulating Mitotic Exit and Cytokinesis in Higher Eukaryotes

G. Montano, J. Rashe, L. Weber, C. B. Shuster; Biology, New Mexico State University, Las Cruces, NM

Maintenance of genomic stability requires that chromosome segregation and cytoplasmic partitioning are coordinated with high fidelity. However, the molecular mechanism by which this occurs in metazoan cells remains unclear. In yeast, Mob1 (Mps-One Binding) protein participates in coordinating mitosis and cytokinesis by serving as an activating subunit for the Dbf2/Sid2 kinases. Mob1 and Dbf2/Sid2 localize to the spindle poles up until anaphase, at which time they re-localize to the actomyosin ring during cytokinesis. In an effort to determine whether Mob1 plays a similar role in higher eukaryotes, we used the yeast two-hybrid (Y2H) system to identify Mob1A binding proteins in human cells. Using human Mob1A as bait, Large Tumor Supressor 2 (Lats2) kinase was identified from a pretransformed Hela cell cDNA library that demonstrated a strong two hybrid interaction. Lats1 and Lats2 share homology with the Dbf2/Sid2 kinases, but there has been no data to date demonstrating that either binds Mob1. Mutant alleles of yeast Mob1 have been described that inhibit Dbf2/Sid2 binding and cause cytokinesis defects *in vivo*, and alteration of these positions in human Mob1A abolished human Lats2 binding. In humans, there are 4 different isoforms of Mob1 (A-D), and preliminary studies indicate that hLats2 preferentially associates with Mob1A. From these studies, it is our hypothesis that Lats2 is the Dbf2/Sid2 homologue in higher eukaryotes, and further studies *in vitro* and *in vivo* will determine whether this is, indeed, the case.

897

### Ace2p Contributes to Fission Yeast Septin Ring Assembly by Regulating *mid2*$^+$ Expression

C. S. Petit, S. Mehta, R. H. Roberts, K. L. Gould; Cell and Developmental Biology, Vanderbilt University, Nasvhille, TN

The fission yeast *Schizosaccharomyces pombe* divides through constriction of an actomyosin-based contractile ring followed by formation and degradation of a medial septum. Formation of an organized septin ring is also important for the completion of *S. pombe* cell division and this event relies on the production of Mid2p. *mid2*$^+$ mRNA and protein accumulate in mitosis. Recent microarray analyses identified *mid2*$^+$ as a target of the Ace2p transcription factor, and *ace2*$^+$ as a target of the Sep1p transcription factor. We report here that Mid2p production is controlled directly by

Ace2p functioning downstream of Sep1p. As expected then, both Sep1p and Ace2p are required for septin ring assembly and genetic analyses indicate that septin rings function in parallel with other Ace2p targets to achieve efficient cell division. Conversely, forced overproduction of Sep1p or Ace2p prevents septin ring disassembly. We find that Ace2p levels peak during anaphase and Ace2p is post-translationally modified by phosphorylation and ubiquitination. Ace2p localizes symmetrically to dividing nuclei and functions independently of the septation initiation network. In future studies, we plan to investigate the role of Ace2p phosphorylation in its regulation.

## Actin-Associated Proteins II (898-918)

898

### Loss of Profilin Confers Tumorigenic Characteristics to Mammary Epithelial Cells

L. Zou,[1] P. Roy[2]; [1]Bioengineering, University of Pittsburgh, Pittsburgh, PA, [2]Bioengineering and Pathology, University of Pittsburgh, Pittsburgh, PA

**Objective:** Profilin has been implied as a tumor suppressor protein based on downregulation of its expression in mammary tumor cells and its ability to reduce tumorigenicity and migration of breast carcinoma when overexpressed in these cells. However, a direct functional relevance of reduced expression of profilin in tumor progression has not been established. The goal of the present work was to determine whether reduced profilin expression confers tumorigenic characteristics to mammary epithelial cells. **Methods:** A small-interfering RNA (siRNA) was used to silence profilin expression in human mammary epithelial cells (HMEC), while the control cells were transfected with a non-targeting oligonucleotide that has no significant sequence homology with any known mouse or human gene. The effects of downregulation of profilin on HMEC proliferation, adhesion and intercellular junctions were determined. **Results:** Nearly 90% reduction in profilin expression was achieved in HMEC by RNA interference. Immunostaining of vinculin showed a dramatic decrease in the number of focal adhesions in HMEC as a result of silencing of profilin. From the immunostaining of beta-catenin, loss of cell-cell junctions was also evident in the siRNA-treated cells. Finally, silencing profilin resulted in approximately two-fold increase in cell proliferation. **Conclusion:** Since loss of cell-cell and cell-matrix adhesions, and increased cell-proliferation are hallmarks for neoplastic transformation, our results for the first time suggest that suppression of profilin expression may actually be functionally linked to the pathogenesis of breast cancer.

899

### Functional Characterization of Profilin in the Migration of Breast Cancer Cells

V. R. Panchapakesa,[1] P. Roy[2]; [1]Bioengineering, University of Pittsburgh, Pittsburgh, PA, [2]Bioengineering and Pathology, University of Pittsburgh, Pittsburgh, PA

**Objective:** We have previously shown that overexpression of profilin reduces the migration of invasive breast cancer cells. Besides actin, profilin also binds to several proline-rich motif (PRM) proteins that are important for cytoskeletal reorganization. The aim of the present work is to determine whether profilin's interactions with PRM proteins are important for the migration of breast carcinoma. **Methods:** We have generated cell lines of MDA-MB-231 that stably express GFP-fused constructs of either profilin or its mutant form (H133S) that is unable to bind to PRM proteins. GFP-expressing cells served as negative control. **Results:** Expression of H133S mutant prevented endogenous profilin's interaction with actin and thus acted as a dominant negative. Transwell migration experiments demonstrated that expression of both profilin and its H133S mutant reduced EGF-directed chemotactic migration of MDA-MB-231 with higher suppression in cell migration achieved when profilin was overexpressed. Timelapse microscopy revealed that H133S mutant cells were able to produce multiple cell protrusions, which however lacked any directional bias, and hence resulted in minimal net cell translocation. Defect in cell translocation was also observed in profilin overexpresser cells. Increased formation of actin filaments and focal adhesions were also evident in these two types of cell lines. **Conclusion:** These results imply that 1) profilin-PRM interactions may be important for directional and spatial regulation of cell protrusion, and 2) profilin-induced suppression of MDA-MB-231 migration may result from excessive actin polymerization and increase in cell adhesion.

900

### Regulation of Actin Dynamics by Direct Interaction Between Profilin and Srv2/CAP

E. A. Bertling, P. K. Mattila, P. Lappalainen; University of Helsinki, Institute of Biotechnology, Helsinki, Finland

The actin cytoskeleton is regulated by a large number of proteins that interact with monomeric and/or filamentous actin. Cyclase-associated-proteins (CAPs) are highly conserved actin monomer-binding proteins that are found in all eukaryotes examined so far. Yeast CAP (also called Srv2/CAP) is a 58 kDa protein consisting of a highly conserved C-terminal ADP-actin binding domain and an N-terminal regulatory domain. In addition, the C-terminal domain of Srv2/CAP contains two proline-rich regions, which have been suggested to bind profilin and/or SH3 domain proteins. We carried out a biochemical analysis of the C-terminal half of yeast Srv2/CAP (amino acids 253-526) to examine the possible interaction with profilin and to elucidate how this interaction alters actin-binding properties of these proteins. GST pull-down and tryptophan fluorescense assays revealed that Srv2/CAP indeed binds profilin with a Kd of 1.3 uM. Site-directed mutagenesis analysis demonstrated that the first proline-rich region (P1) of Srv2/CAP is essential for profilin-binding, whereas the second proline-rich region (P2) does not contribute to interactions with profilin. Our fluorometric binding assays also demonstrated that profilin-binding to Srv2/CAP does not interfere with actin-interactions of Srv2/CAP, suggesting that these proteins can form a ternary complex consisting of Srv2/CAP, profilin, and two actin monomers. In our mutagenesis studies, we also identified one Srv2/CAP allele that interacts with ADP-actin monomers with normal affinity but has strong defects in profilin-binding. We are currently examining the biological role of profilin-Srv2/CAP interaction by analyzing the effects of this mutant Srv2/CAP when expressed in yeast instead of wild-type protein.

901

### Silencing Profilin Stabilizes Intercellular Junctions of Vascular Endothelial Cells

Z. Ding,[1] P. Roy[2]; [1]Bioengineering, University of Pittsburgh, Pittsburgh, PA, [2]Bioengineering and Pathology, University of Pittsburgh, Pittsburgh, PA

**Objective:** Vascular hyper-permeability as a result of compromised integrity of intercellular junctions of endothelial cells (EC) contributes to

pathogenesis of many life-threatening disorders. Permeability mediators stimulate assembly of actin stress fibers (SF) and formation of focal adhesions (FA) in EC. These steps requiring actin polymerization are critical for generating transcellular contractility, which is partly responsible for junctional disruption and loss of EC barrier function. As profilin is thought to be an important catalyst of actin polymerization, we examined whether silencing profilin improves the junctional stability of EC. **Methods:** A small interfering RNA (siRNA) was used to silence profilin expression in human umbilical vein endothelial cells (HUVEC), while the control cells were transfected with a non-targeting oligonucleotide that has no significant sequence homology with any known mouse or human gene. **Results:** Nearly 100% silencing of profilin expression was achieved in EC by RNA interference. Immunostaining of ZO-1, VE-cadherin and beta-catenin revealed that EC after siRNA treatment became resistant to junctional disruption in response to VEGF treatment. An increase in VE-cadherin expression was observed in siRNA expressing cells. Furthermore, silencing profilin prevented VEGF-induced assembly of actin SF and FA in EC. Finally, data from wound-healing experiments showed that suppression of profilin significantly inhibited EC migration, an event that also widens the gap between neighboring EC during vascular hyper-permeability. **Conclusions:** Taken together, these results suggest that silencing profilin may be an effective strategy to reduce endothelial permeability.

902

### Two Mouse Cofilin Isoforms, Muscle-type (MCF) and Non-muscle Type (NMCF), interact with F-actin by Different Efficiency

K. Nakashima,[1] N. Sato,[1] T. Nakagaki,[1] H. Abe,[1] S. Ono,[2] T. Obinata[1]; [1]Department of Biology, Chiba University, Chiba-shi, Japan, [2]Department of Pathology, Emory University, Atlanta, GA

Two cofilin isoforms, muscle-type (MCF) and nonmuscle-type (NMCF), are co-expressed in developing mammalian skeletal and cardiac muscles. MCF is expressed more abundantly in slow skeletal muscle than in fast skeletal muscle. To clarify how they are involved in actin filament dynamics during myofibril assemly, we examined their localization in muscle tissues and cultured muscle cells by immunocytochemical methods with the antibodies specific for MCF or NMCF and their interaction with F-actin in vitro. NMCF was detected mostly in a diffused pattern in the cytoplasm but MCF was partly localized to the striated structures in myofibrils. Location of chicken cofilin, a homologue of MCF, in I-bands of myofibrils was determined by an immunocytochemical method. The results suggest that MCF is associated with actin filaments in muscle cells more efficiently than NMCF. Using purified recombinant MCF and NMCF, their interaction with F-actin was examined in vitro by cosedimentation assay. We observed that MCF was precipitated with F-actin more effectively than NMCF. When MCF and NMCF were simultaneously incubated with F-actin, MCF was preferentially associated with F-actin. MCF and NMCF inhibited interaction of F-actin with tropomyosin, but the former suppressed the actin-tropomyosin interaction more strongly than the latter. These results suggest that MCF interacts with F-actin with higher affinity than NMCF, and although both of them are involved in the regulation of actin assembly in developing myotubes, the two proteins may play somewhat different roles

903

### Cofilin and Phalloidin Co-rescue Filament Formation in Polymerization-deficient Actin Mutant

A. Shvetsov,[1] V. Galkin,[2] A. Orlova,[2] M. Phillips,[1] P. Rubenstein,[3] E. Egelman,[2] E. Reisler[1]; [1]Department of Chemistry and Biochemistry and Molecular Biology Institute, UCLA, Los Angeles, CA, [2]Department of Biochemistry and Mol. Genetics, University of V.A. Jordan Hall, Charlottesvile, VA, [3]Department of Biochemistry, University of Iowa College of Medicine, Iowa City, IA

Previous studies demonstrated that cofilin, which is essential for actin filament turnover in vivo, changes the helical twist and weakens longitudinal and lateral contacts in F-actin. Phalloidin stabilizes these lateral and longitudinal contacts and competes with cofilin for the binding to F-actin. Using yeast actin triple mutant, L180C/L269C/C374A, we showed previously that locking the hydrophobic loop to G-actin surface by a disulfide bridge prevents filament formation. Neither phalloidin nor cofilin alone can induce the polymerization of this actin. Myosin subfragment 1 is equally inefficient in this task. Here, we show that together cofilin and phalloidin co-rescue the polymerization ability of the polymerization-deficient cross-linked actin. EM revealed a dense contact between opposite strands in F-actin, suggesting cofilin-phalloidin-induced conformational changes in actin protomers that support interstrand interactions. EM analysis showed that cofilin changes the helical twist of the disulfide cross-linked actin filaments from 166 to 165 degrees, indicating either partial or disordered attachment of cofilin to F-actin and/or a competition between cofilin and phalloidin to alter F-actin symmetry. We propose that there are regions of F-actin primarily occupied with cofilin, which bridges between longitudinal protomers, followed by regions where phalloidin stabilizes lateral and longitudinal contacts.

904

### Role of the Actin Binding Protein Cofilin/Adf in Cell Migration and Mouse Development

W. Witke, C. Gurniak, G. Bellenchi, E. Perlas; Mouse Biology Unit, EMBL, Monterotondo, Italy

Cofilin and ADF belong to the family of F-actin depolymerizing factors thought to be essential for regulating actin polymerization and directed cell migration. Unlike lower eukaryotes, three cofilin/ADF genes are found in the mouse - non-muscle cofilin (n-cofilin), muscle cofilin (m-cofilin) and ADF. We have generated mouse models for n-cofilin and ADF applying conventional as well as conditional mutagenesis. Using these mouse models as well as cells cultured from the respective mutants we have been able to distill specific roles of the depolymerizing factors in cell migration, proliferation and cell cycle control. Recent results on n-cofilin/ADF function in different tissues and cell types using cre-lox mediated deletion will be discussed.

905

### *Salmonella* SipC Nucleates Actin to Promote Bacterial Entry

J. Chang, J. Chen, D. Zhou; Biological Sciences, Purdue University, West Lafayette, IN

*Salmonella* type III secreted SipC possesses dual effector translocation and actin-modulating functions. The biological significance of SipC's actin nucleation activity in *Salmonella*-induced actin cytoskeleton rearrangements and bacterial invasion has not been studied. We report here the delineation of the actin nucleation activity from its effector translocation activity. Our data showed that the central amino acid 201-220 region is essential for its actin nucleation activity and the amino acid 321-409 region is required for its effector translocation activity. A SipC nucleation deficient mutant, which maintained its effector translocation activity, was obtained. This nucleation-deficient mutant had significantly reduced

ability to induce actin cytoskeleton rearrangements resulting in lower bacterial invasion into HeLa cells. Contrary to previous report, we found that the purified recombinant wild type $SipC_{199-409}$ protein is monomeric in solution by size exclusion chromatography coupled with multiangle laser light scattering assays (SEC-LS). Our data established that the actin nucleation activity of SipC plays a vital role in *Salmonella*-induced membrane ruffles and subsequent bacteria invasion.

906

**Cortical and Gap Junction Distribution of Human Titin Carboxyl Terminus and Co-localization With Actin**

J. Qi,[1] L. Chi,[1] A. J. Banes[2,1]; [1]Flexcell International Corporation, Hillsborough, NC, [2]Department of Biomedical Engineering, Curriculum in Applied and Material Sciences, University of North Carolina, Chapel Hill, NC

**Introduction** - Titin, as a major structural protein in sarcomeres, interacts with many of sarcomeric proteins such as myosin, actin, α-actinin, titin-cap (telethonin), calpain, obscurin and MURFs. Most of these proteins, except MURFs which were linked to M-band of titin, bind to Z-band of titin. Few titin partner proteins were located in the carboxyl (C-) terminus of titin. Since titin was also reported to express in non-muscle tissues and cells, its distribution in these tissues and cells will contribute to the fully understanding the functions of titin. In this study, we investigated the intracellular localization of C-terminal titin (M7-10) in COS-7 cells and its interaction with cytoskeletal proteins. **Methods** - The human C-terminal titin cDNA was amplified from total human skeletal muscle cDNA and cloned into pcDNA3 vector. The construct was transfected into COS-7 using lipofectamine. The transiently expressed titin C-terminal fragments were stained with anti-titin rabbit polyclonal antibody M8M9 which recognizes titin C-terminal M8-M9 domains. The stained C-terminal titin was visulized with goat anti-rabbit IgG Alexa 568. Actin fibers were co-stained with BODIPY FL-phallacidin. **Results** - The result showed cortical and gap junction distribution of the C-terminal titin fragments heterogenously expressed in COS-7 cells and co-localized with actin. The immunostaining of mock-transfected COS-7 cells with M8M9 antibody did not reveal any endogenous signal, this confirmed the linkage between C-terminal titin fragments and actin. **Conclusion** - The results in this study indicate that actin may bind to titin in its C terminus directly or indirectly through adaptor proteins. This finding will facilitate the understanding the cellular functions of titin in non-muscle tissues and cells.

907

**The Formation of GFP-Tagged Hirano Bodies in *Dictyostelium discoideum* After Inducible Expression of a Truncated Actin Bundling Protein**

J. F. Reyes, A. G. Maselli; Biological Sciences, Chicago State University, Chicago, IL

Neurodegenerative disease such as Alzheimer's disease and Pick's disease have been associated with unique protein aggregates including neurofibrillary tangles, Lewy bodies and amyloid plaques, but little attention has been given to Hirano Bodies (HB). Hirano Bodies are rod-like cytoplasmic inclusions of actin and actin associated proteins. The formation of Hirano bodies has been shown to occur in *Dictyostelium discoideum* after expression of a truncated form of the 34-kDa actin bundling protein, providing a unique opportunity to study protein aggregation in living cells. We have developed a system to monitor the formation of HB using a vector with inducible promoter activity. The ribonucleotide reductase promoter (Gaudet et. al. 2001) is a strong inducible promoter with an activity lasting 3 hours post induction. To visualize the formation of Hirano Bodies, Green Fluorescent Protein (GFP) was fused to the carboxyl terminal end of the truncated form of the 34-kDa protein. Here, we report a 35-fold increase in the presence of GFP-tagged, rhodamine phalloidin positive cytoplasmic inclusions after promoter induction with UV light. Our results suggest that a short pulse of promoter activity produces sufficient protein to begin the formation of Hirano Bodies. The inclusions observed by GFP vary considerably but the average area is 5.7 $\mu m^2$ , SD ± 11.6 $\mu m^2$ in the induced populations. The measurements were obtained 15 hours post induction when the inclusions formed can be easily observed. Using this inducible system will allow us to further study Hirano Bodies and characterize the kinetics and their fate within living cells.

908

**The G35 Antigen Appears to be a Form of α-actinin Which Co-isolates and Co-localizes with Type III Intermediate Filaments**

A. Abedi, D. M. García, J. R. Koke; Department of Biology, Texas State University-San Marcos, San Marcos, TX

Monoclonal antibody G3.5 was raised against multiple sclerotic plaque material from human brain and spinal cord. The antigen of this antibody has been previously designated an α-actinin-like protein with the unusual features of localizing separately from skeletal muscle α-actinin, localizing with desmin in muscle and with GFAP in astrocytes, and simultaneously binding actin and desmin *in vitro*. The physical properties of the antigen are essentially identical to the published properties of α-actinin, and initial amino acid sequencing showed a high degree of identity to sarcomeric α-actinin. Attempts to use amino acid sequence data to isolate a unique gene sequence for the G3.5 antigen have failed. Recent studies have demonstrated that α-actinin has a more complex role in cellular structure and development than previously thought. α-actinin has been shown to be subject to tyrosine kinase phosphorylation and associate with many proteins in addition to actin. Because of this new information, we reisolated the G3.5 antigen from rat skeletal muscle as before, using a modified desmin isolation protocol. The G3.5 antigen was then released and separated from the insoluble desmin fraction by addition of a reducing agent and centrifugation. Limited proteolysis and sequencing of the G3.5 antigen has provided 7 fragments totaling 72 residues, 98.6% identical in sequence to regions found in rat sarcomeric α-actinin. Immunohistochemical analysis of the G3.5 antigen was also performed and produced results similar to those described above. The preponderance of evidence therefore suggests that either the G3.5 antigen is a novel form of α-actinin that associates with type III intermediate filaments as well as actin, or that the mAB G3.5 reveals a previously undescribed association or binding site on type II α-actinin for type III IFs.

909

**Palladin Binds Directly to F-actin and Crosslinks Actin Filaments**

A. S. Rachlin,[1] M. J. Costello,[2] C. A. Otey[1]; [1]Cell and Molecular Physiology, University of North Carolina, Chapel Hill, NC, [2]Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC

Palladin is a multi-isoform protein that localizes to actin-rich structures, including stress fibers, focal adhesions, cell-cell junctions and the Z-line of cardiac myocytes. Palladin's role as a cytoskeletal scaffold is supported by an emerging list of cytoskeletal proteins with which it interacts. This group includes the f-actin crosslinking protein alpha-actinin, VASP and profilin, proteins with well-defined roles in actin polymerization and the

protein Lasp-1, which plays an important role in cellular motility. All of the above mentioned proteins have been shown to directly bind filamentous and/or monomeric actin. The overexpression of palladin in a variety of cultured cells induces the formation of unusually large, hyper-bundled stress fibers. We set out to determine if these bundles are generated indirectly, as the result of palladin's interactions with its binding partners, or if palladin may have direct effects on f-actin organization. To address this question, baculovirus expressed palladin was purified and a co-sedimentation assay was used to determine if palladin binds directly to f-actin. When palladin was mixed with skeletal muscle actin in a 1:10 molar ratio, the bulk of palladin co-fractionated with f-actin. In addition, purified palladin was found to crosslink individual actin filaments into bundles, as observed by fluorescence microscopy and differential centrifugation. Palladin induced actin bundles were further analyzed using transmission electron microscopy. These results suggest that the overexpression phenotype of actin bundling is due in part to palladin's own independent crosslinking activity and suggest a role for palladin in the contractile machinery of the cell. Taken together, the evidence to date indicates that palladin is both an actin cross-linking protein and a scaffold for the recruitment of other actin-binding proteins of widely varying activities, suggesting that palladin may occupy an unusual functional niche in regulating actin assembly in cells.

910

**Biochemical Studies on Mouse Inverted Formin 2 (INF2)**

E. Seth Chhabra, H. N. Higgs; Biochemistry, Dartmouth Medical School, Hanover, NH

**Background:** Formins are a conserved class of eukaryotic proteins which regulate actin dynamics. There are seven metazoans formin groups, which include 15 mammalian formin genes. These proteins are characterized by the presence of FH1 and FH2 (formin homology 1 and 2) domains. Inverted Formins (INF) are members of a recently identified unique formin group which is present in metazoans only. The FH1 and 2 domains in these formins are present at the N-terminus while in all other formin groups these domains are at the C- terminus. **Objective:** We are examining the biochemical effects of mouse inverted formin 2 (INF2) on actin polymerization dynamics. **Results:** An N- terminal construct (INF2-N), containing FH1 and 2 domains, possesses properties similar to those of other formins, including: nucleation acceleration; elongation inhibition (90% inhibited); profilin- mediated relief of elongation inhibition; and antagonism of complete barbed end capping by heterodimeric capping protein. A C-terminal construct binds one actin monomer, mediated most likely by a WH2 motif, resulting in monomer sequestration. Full length protein (INF2-Full) has surprising properties. While it accelerates assembly of actin filaments from ATP-monomers, it also causes disassembly of actin filaments when phosphate release is not inhibited. This dual activity leads to biphasic polymerization dynamics, with initial filament assembly followed by disassembly, and has possible cellular implications. **Conclusion:** The dual assembly/ disassembly properties of INF2 provide interesting possibilities for cellular regulation of actin dynamics.

911

**Glutathionylation of Mammalian β,γ-F-actin Enhances its Interaction with Human Cofilin**

L. A. Selden,[1] R. L. Finkle,[1] J. S. Dengler,[1] H. J. Kinosian,[1] L. C. Gershman,[1] J. E. Estes[1,2]; [1]Research Service, Stratton VA Medical Center, Albany, NY, [2]Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY

Cytoskeletal rearrangements accompany the cellular response to oxidative stress and involve the actin binding protein cofilin. Reversible glutathionylation of actin may play a role in modulating this response. We have used F-actin containing a small amount (≤ 10%) of pyrene-labeled actin to monitor the binding of human cofilin to mammalian β,γ-nonmuscle actin. The pyrene-actin fluorescence is quenched by cofilin binding, but the actin subunits containing pyrene cannot be glutathionylated due to the presence of pyrene at the glutathionylation site (cysteine 373). Thus, differences between control and glutathionylated actin represent changes in the pyrene-actin environment rather than a direct effect of glutathionylation on the actin subunit. Working at pH 6.5 and low concentrations of F-actin (0.5 μM) and cofilin (1 μM), we found that pyrene fluorescence quenching by cofilin was slow and incomplete for control samples. Glutathionylation of the actin increased the initial rate of quenching by nearly 50-fold. Removal of the glutathione from the actin with DTT restored the quenching rate to that of the control. These data suggest that the reversible glutathionylation of actin can modulate the binding of cofilin to actin and may contribute to the rearrangement of the actin cytoskeleton in response to oxidative stress. Supported by the Department of Veterans Affairs.

912

**Fascin1-deficient Mice Show Neurological Defects**

Y. Yamakita, S. Yamashiro; MBB, Rutgers Univ., Piscataway, NJ

Fascin is an actin-bundling protein, and is believed to be involved in the assembly of actin bundles in filopodia. Vertebrates have three fascin genes (fascin1-3). While expression of fascin2 and fascin3 is strictly restricted to retina and testis, respectively, fascin1 is more ubiquitously expressed and particularly abundant in cells with well-developed filopodia such as neuronal cells. Because filopodia are critical for cell motility and/or interactions with other cells, fascin1 has been suggested to play roles in growth cone motility and guidance of neuronal cells. To explore these possibilities, we generated fascin1 knockout (KO) mice. Disruption of fascin1 gene was confirmed by southern blotting, as well as PCR-based genotyping and Western blotting. RT-PCR analyses revealed that other fascin gene products (fascin2 and fascin3) did not compensate fascin1 expression. While fascin1-deficient mice were viable and fertile, they showed neurological defects, as judged from the following phenotypes: First, tail suspension tests conducted with 3 months or older mice revealed that fascin1 KO mice held their limbs into their bodies (so-called "huggers" phenotype) whereas wild-type mice spread their limbs. Second, more than 50% of fascin1-deficient mice showed spontaneous seizures. Nissl staining of coronal brain sections revealed that the posterior of anterior commissure neurons was mostly lacking in fascin1 KO mice, suggesting that fascin1 deficiency may affect extension of anterior commissure neurons. We also examined, using dorsal root ganglionic neurons (DRGNs), whether fascin1 deficiency affects growth cone morphology and neurite outgrowth. We found that DRGNs from either fascin1 KO or wild-type mice were able to extend neurites. However, the size of growth cones of fascin1-deficient DRGNs is smaller and filopodia are less in number and shorter in length. Our results suggest that vertebrate fascin1 affects growth cone dynamics and is critical for proper formation of neuronal networks.

913

**The Actin Filament Crosslinker Fascin 2 Localizes to the Inner Segment Actin Filament Bundles of Photoreceptors**

J. Lin-Jones, B. Burnside; Molecular and Cell Biology, University of California - Berkeley, Berkeley, CA

Fascin 2 is an actin-filament cross-linker that is expressed exclusively in the retina. Fascin 2 is important for the maintenance of functional photoreceptors as a human mutation in fascin 2 results in autosomal dominant retinitis pigmentosa and macular degeneration. While fascin 2 has been localized to photoreceptors in the retina, its exact subcellular localization has not been determined. The purpose of this study is to examine in more detail the location of fascin 2 in vertebrate photoreceptors. We have cloned fascin 2 cDNA from Xenopus and zebrafish retinal libraries and used bacterial fusion proteins to Xenopus and zebrafish fascin 2's to generate antibodies. In addition, Xenopus fascin 2 fused to green fluorescent protein has been expressed in rod photoreceptors in transgenic Xenopus tadpoles to examine the subcellular localization of fascin 2 in rods. Results from both immunohistochemistry and transgenic tadpoles using fluorescence microscopy demonstrate that fascin 2 is localized to inner segment actin filament bundles and calycal processes that encircle the proximal outer segment of photoreceptors. Thus, the data suggest that fascin 2 plays an important role in maintaining the structural actin cytoskeleton of photoreceptors by affecting the actin filament bundles of the photoreceptor inner segment.

914

**Characterization of Drebrin-A: A Spine Specific Actin-bundling Protein Which Competitively Binds to F-actin with Tropomyosin and Caldesmon**

R. Ishikawa, A. Takahashi, Y. Hanyuda, A. Nakamura, K. Kohama; Department of Pharmacology, Gunma University School of Medicine, Maebashi, Japan

Drebrin is an actin-binding protein specifically expressed in brain. Two types of isoforms, embryonic type (drebrin-E), and adult type (drebrin-A) are produced from a single gene by alternative splicing. Drebrin-A localizes in dendritic spine of mature neuron, and suggested to affect spine morphology. In this report, we successfully purified drebrin-A by using bacterial expression system, and characterized in vitro. Drebrin-A bound to actin filaments with a stoichiometry of 4 actin molecules to 1 drebrin-A molecule. It inhibited the actin-binding activity of caldesmon by 70%. This inhibitory effect was enhanced in the presence of tropomyosin. Furthermore, caldesmon coupled with tropomyosin inhibited the actin-binding of drebrin, indicating that drebrin competitively binds to F-actin with tropomyosin and caldesmon. Low speed centrifugation assay revealed that drebrin-A had actin-crosslinking activity, which were confirmed by direct visualization of fluorescently labeled F-actin complexed with drebrin-A. These results suggest that caldesmon coupled with tropomyosin may affects spine morphology through regulating drebrin-A activity.

915

**Regulation of Villin-Induced Cell Migration by Lysophosphatidic Acid**

A. Tomar,[1] S. George,[1] N. Kumar,[1] I. G. Macara,[2] S. Khurana[1]; [1]Physiology, University of TN, Memphis, TN, [2]Center for Cell Signalling, University of Virginia, Charlottesville, VA

Villin is an epithelial cell-specific protein that nucleates, severs, caps and cross-links actin filaments. The actin-modifying activities of villin are regulated in vitro by calcium, phosphoinositides and tyrosine phosphorylation. In this study, we demonstrate for the first time the association of villin with lysophosphatidic acid (LPA). LPA binds to previously identified phosphatidylinositol 4,5-bisphosphate binding sites in villin, however the two ligands have opposite effects on the actin modifying activities of villin. The direct association of LPA with villin inhibits actin-capping, -severing, -polymerizing and -bundling activities of villin but enhances c-src mediated tyrosine phosphorylation of villin. In contrast, the direct association of villin with $PIP_2$ enhances the actin-bundling activity of villin and inhibits c-src mediated tyrosine phosphorylation of villin. Using time-lapse video microscopy, MDCK Tet-Off cells transfected with yellow fluorescent tagged villin protein (SEYFP-villin), we show that LPA enhances cell migration and the formation and growth of lamellipodia in cells overexpressing SEYFP-villin. This was accompanied by the redistribution of villin to the leading edge. LPA-induced cell migration in these cells is completely inhibited by the PLC inhibitor U-73122 but not its inactive analog U-73144 nor by the $G\alpha_i$ inhibitor, pertusis toxin. MDCK cells express the LPA receptor, LPA2. Our hypothesis is that LPA can modulate villin-induced cell migration either by directly associating with villin (intracellular regulation) or by activation of G protein-coupled receptor LPA2. Phospholipids like LPA can be safely added to a regular diet and their levels can be modulated by various pharmacological agents leading us to speculate that LPA-regulated intestinal cell migration by villin can have therapeutic benefits.

916

**Expression of Full-length Smooth Muscle Myosin Light Chain Kinase**

A. Nakamura,[1] K. Kawano,[1] Y. Gao,[2] Y. Hanyuda,[1] A. Takahashi,[1] R. Ishikawa,[1] K. Kohama[1]; [1]Department of Molecular and Cellular Pharmacology, Gunma University Graduate School of Medicine, Maebashi, Japan, [2]Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian, China

Myosin light chain kinase (MLCK), family of calmodulin-dependent protein kinases, catalyzes the phosphorylation of regulatory light chain (MLc 20) of myosin, and plays an important role in activating actomyosin-linked contractility in smooth muscle cell[1)2)]. In spite of the trial to express full-length MLCK by various expression systems, it has not been successful yet. So, we tried to make a recombinant bovine stomach MLCK (BsMLCK) by using a new bacterial expression system, which was developed by Prof. Masayori Inouye of Robert Wood Johnson Medical School[3)]. We succeeded in the high level expression (5-10mg/L culture medium) of full-length BsMLCK by using the cold shock bacterial expression system. Recombinant BsMLCK was a soluble form and phosphorylated MLc20. It also had actin-binding and -bundling activities. Recombinant BsMLCK with the properties known for the MLCK was expressed in the large amount, which should be the first step to analyze the structure and function of MLCK. [References] 1. Hayakawa, K., Okagaki, T., Ye, LH., Samizo, K., Higashi-Fujime, S., Takagi, T., and Kohama, K. (1999) *Biochim. Biophys. Acta.* 1450, 12-24 2. Gao, Y., Ye, LH., Kishi, H., Okagaki, T., Samizo, K., Nakamura, A., and Kohama, K. (2001) *IUBMB Life.* 51, 337-344. 3. Qing, G., Ma, LC., Khorchid, A., Swapna, GV., Mal, TK., Takayama, MM., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, GT., Ikura, M., and Inouye, M. (2004) *Nat. Biotechnol*. 22, 877-882.

917

**Ezrin Is Activated by PKC and Contributes to Metastatic Phenotype of Osteosarcoma Cells**

L. Ren, S. Hong, J. Cassavaugh, C. Khanna; Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD

Osteosarcoma (OSA) is the most common primary tumor of bone. Despite successful control of the primary tumor and adjuvant chemotherapy,

death from pulmonary metastases occurs in over 30% of patients within 5 years. Using a genomic approach we identified ezrin, a membrane-actin cytoskeleton linker protein, to be over-expressed in the more aggressive K7M2 murine OSA model compared to the less aggressive K12 model. In vivo studies in this murine model demonstrated that ezrin protein and ezrin function were necessary for metastasis. Furthermore, studies in child-hood OSA patients suggested that high primary tumor expression of ezrin was associated with a shortened disease free interval compared to patients with low primary tumor expression ($p<0.01$). Phosphorylation of C-terminal threonine 567 on ezrin is believed to be important for ezrin activation. The kinase responsible for ezrin activation at this C-terminal threonine in vivo and particularly during metastasis of cancer is not known. Using pharmacological inhibitors we found that phosphorylation of ezrin at T567 was dependent on PKC (BIM, Ro31-8220, Go6976) but not by inhibition of Rho-kinase (Y27632) or PI3 Kinase (LY294002). To investigate the role of PKC and ezrin in regulation of tumor metastasis, wound closure (cell migration) assays were performed using K7M2 cells. Cell migration was inhibited by both PKC inhibitor Go6976 and through the antisense suppression of ezrin. Studies are underway to determine if PKC controled cell migration is mediated through or dependent on ezrin.

918

### Deregulated RhoA Signaling is Detrimental to Arp2 mRNA Localization

L. A. Mingle, G. Liu; Cell Biology & Cancer Research, Albany Medical College, Albany, NY

The Arp2/3 complex, a nucleator of actin polymerization, is localized to the leading edge of migrating cells. The mRNAs for the subunits of the complex also localize to the leading protrusions of fibroblasts, suggesting that mRNA localization may mediate the protein complex localization. The localization of Arp2 mRNA, one subunit of the complex, is dependent on external stimuli. However, the signal transduction mechanisms mediating this localization remain unknown. Here we present evidence that a signaling pathway utilizing RhoA is important for Arp2 mRNA localization. Using chicken embryo fibroblasts (CEFs), we show that Arp2 mRNA localizes to the leading protrusions in CEFs in response to platelet derived growth factor (PDGF-BB) and basic fibroblast growth factor (bFGF) stimulation. However, neither activators nor inhibitors for Protein kinase C (PKC) or Protein kinase A (PKA) could significantly alter the Arp2 mRNA localization, suggesting that PKC and PKA are not involved in Arp2 mRNA localization under these conditions. In contrast, Rho kinase (ROCK) is required for Arp2 mRNA localization, as pretreatment with Y-27632 significantly inhibits serum induced Arp2 mRNA localization. Interestingly, Arp2 mRNA localization is also inhibited by the constitutive activation of R-Ras and RhoA. Constitutive activation of either R-Ras or Rho has no effect on β-actin mRNA localization, suggesting that Arp2 mRNA localization may utilize a unique mechanism. These data suggest that Rho signaling is necessary for Arp2 mRNA localization, but that too much or too little RhoA activity is detrimental to the localization. We are currently testing if RhoA needs to be spatially regulated for Arp2 mRNA to localize to the leading protrusion.

## Actin Dynamics & Assembly I (919-938)

919

### A Primary Role for the Second SH3 Domain of the Eukaryotic Adaptor Protein Nck in Actin Assembly Induced by Enteropathogenic *E coli*

P. J. Savage, L. Magoun, J. M. Leong; Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA

Upon infection of the human intestine, enteropathogenic E. coli (EPEC), reorganizes the host cell cytoskeleton to generate filamentous actin-rich structures, known as pedestals, beneath the sites of bacterial attachment. To trigger actin assembly, EPEC translocates the bacterial protein Tir into the host cell, where it is clustered at the plasma membrane and phosphorylated at tyrosine residue 474. The Tir sequence encompassing phosphorylated Y474 recruits Nck, a eukaryotic adaptor protein comprised of three SH3 domains and one SH2 domain, to initiate actin pedestal formation in an N-WASP-dependent fashion. Latex beads coated with a twelve amino acid Tir peptide containing phosphorylated Y474 recruit Nck and stimulate localized actin assembly in cell-free extracts. To determine which domains of Nck are critical for function, we tested Nck deletion derivatives for the ability to promote actin assembly around latex beads coated with Tir peptide in cell-free mammalian extracts. As expected, the C-terminal SH2 domain, which binds the Tir phosphopeptide, was essential for function. In addition, a fragment carrying the second and third SH3 domains of Nck, in conjunction with the SH2 domain, was able to promote actin assembly in vitro. The lack of a role for the first SH3 domain was confirmed by complementation of pedestal formation by Nck-deficient cells with Nck deletion derivatives. In fact, whereas a Nck derivative carrying the SH2 and the third SH3 domains promoted only moderate levels of assembly, an analogous fusion carrying the SH2 and the second SH3 domains restored pedestal formation to wild-type levels. These results indicate that of the three SH3 domains of Nck, only the second SH3 domain plays an essential role in EPEC-induced actin assembly.

920

### Molecular Characterization of the T maritima MreB

G. J. Bean,[1] J. A. Mayer,[2] L. Hazelwood,[3] D. Anjum,[3] D. Hanein,[3] K. J. Amann[4]; [1]Molecular Biology, UW-Madison, Madison, WI, [2]Cell and Molecular Biology Program, UW-Madison, Madison, WI, [3]Burnham Institute, La Jolla, CA, [4]Zoology / Molecular Biology, UW-Madison, Madison, WI

Bacterial actin homologues play critical roles in the determination of cell shape and DNA segregation. Little is currently known about the biochemical functions of these molecules, including their capacities for polymerization dynamics and intermolecular interactions. We are studying these properties of several prokaryotic actin homologues with the intention of explaining the manner in which the bacterial cytoskeleton carries out its physiological role. We have expressed in *E. coli* and purifed the actin homologue MreB from the thermophile *T. maritima.* As measured by light scattering and sedimentation, MreB polymerizes in the presence of divalent cations, as do eukaryotic actins. It binds ATP and rapidly hydrolyzes it rapidly following polymerization. In many ways, however, MreB exhibits unexpected properties for an actin homologue. Its polymerization is highly sensitive to pH, ionic strength, and temperature changes. The products of polymerization can be sedimented at much lower centrifugal forces than can individual actin filaments. By electron microscopy, at least two different structures can be produced by manipulation polymerization conditions: very thin, short stubs; and tube-like structures that are reminiscent of microtubules. We suggest that MreB nucleation and polymerization mechanisms are highly distinct from those of eukaryotic actins and that their physiological functions are likely to differ greatly as well.

921

**Molecular Mechanism of Rickettsia Actin-Based Motility**

R. L. Jeng,[1] E. D. Goley,[1] J. A. D'Alessio,[1] T. M. Svitkina,[2] O. Y. Chaga,[3] R. A. Heinzen,[4] G. G. Borisy,[3] M. D. Welch[1]; [1]Dept. of Molecular and Cell Biology, UC Berkeley, Berkeley, CA, [2]Dept. of Biology, University of Pennsylvania, Philadelphia, PA, [3]Dept. of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL, [4]Laboratory of Intracellular Parasites, NIAID, NIH, Rocky Mountain Laboratories, Hamilton, MT

Spotted fever group *Rickettsia* are obligate intracellular pathogens that cause a range of diseases such as Rocky Mountain spotted fever. During infection, bacteria exploit the host-cell actin cytoskeleton to promote entry, motility, and cell-to-cell spread, but little is known about the molecular components involved in these processes. Several observations suggest that the underlying mechanisms behind *Rickettsia* motility differ from those involved in the motility of other pathogens such as the well-studied *Listeria monocytogenes*. In particular, *L. monocytogenes* generates actin comet tails containing branched filaments using an Arp2/3 complex-dependent mechanism while *Rickettsia* generates comet tails consisting of unbranched filament arrays that lack the Arp2/3 complex. To better understand the molecular mechanism of *Rickettsia* motility, we identified a candidate nucleating factor called RickA that exhibits sequence similarity to the WASP-family of proteins. RickA is similar to other WASP-family proteins in its ability to stimulate the nucleation and branching activities of the Arp2/3 complex *in vitro*. When coated on the surface of micrsospheres, RickA is also sufficient to promote motility and generate comet tails in cell-free extracts. However, we found that RickA is localized to the bacterial cytoplasm rather than at the cell surface where an actin nucleator would be expected to function. Furthermore, by itself, RickA generates branched filament arrays. These results suggest that, while RickA may be integral to actin nucleation at the surface of *Rickettsia*, other factors are likely to be involved in the nucleation and organization of filaments. To identify some of these factors, we examined two candidate proteins that appear to be involved in organizing actin filaments in *Rickettsia* comet tails: the actin bundling protein, fascin, and an F-actin binding protein, VASP. Both proteins localize to *Rickettsia* tails, and we are currently testing their contribution to *Rickettsia* motility using RNA-interference techniques.

922

**Interaction Between Prokaryotic Actin ParM and the R1-plasmid Kinetechore Complex ParR/parC: Force Generation, Polarity, and Insertional Polymerization**

E. C. Garner,[1] H. G. Garcia,[2] L. Coin,[2] L. Trichet,[2] D. R. Mullins[1]; [1]Biochemistry, UCSF, San Francisco, CA, [2]Physiology course, Marine Biological Labs, Woods Hole, MA

The R1 par operon is thought to construct a minimal DNA-segregating spindle from three components, the centromeric DNA sequence parC, the DNA binding protein ParR, and the actin homolog ParM. In vivo, the ParR/C complex is thought to couple ParM polymerization to plasmid segregation. In vitro, ParM filaments exhibit rapid spontaneous nucleation, symmetrical elongation, and dynamic instability. Using Total Internal Reflection Fluorescence (TIRF) microscopy, we show that the ParR/parC complex stabilizes individual ParM filaments against catastrophic depolymerization. ParR/parC-mediated stabilization produces a population of long filaments that grow asymmetrically with one fast-growing and one slow-growing end. The fast-growing end elongates by insertion of ParM monomers at the interface with ParR/parC. To demonstrate that the connection between ParM and the ParR/parC complex is capable of generating force, we coupled ParR/parC complexes to micron-sized polystyrene spheres and incubated them with ParM in the presence of non-hydrolyzable nucleotides. Assembly of ParM filaments occurs preferentially at the surface of the microsphere and filaments are rapidly organized into an asymmetrical bundle. Elongation of filaments at the interface with ParR/parC generates force propelling the microshpere through the medium. By polarization microscopy the filaments in the bundle are aligned parallel to the direction of motion. By confocal fluorescence microscopy the bundle has a hollow core. Based on these results, we propose a model for assembly and function of a DNA-segregating ParM spindle.

923

**In vivo Dynamics of the Bacterial Actin ParM**

C. S. Campbell, D. Mullins; University of California San Francisco, San Francisco, CA

The R1 Par operon is a self-contained plasmid partitioning system composed of three parts: *parC*, ParR and ParM. *parC* is a stretch of DNA consisting of 10 sequential repeats, each of which binds ParR. The ParR/*parC* complex in turn binds the actin homolog ParM. Previous studies have shown that ParM forms filaments nearly identical to those of eukaryotic actin filaments and that ParM filament bundles appear to position plasmids at each end of a rod-shaped cell. We recently demonstrated that ParM filaments are dynamically unstable and can elongate bidirectionally *in vitro*. These observations led to a model in which ParM filaments continually search the cytoplasm and eventually capture a ParR bound *parC* region on a plasmid. Insertional polymerization at the ParM/ParR interface will then push the plasmids to opposite ends of the cell and hold them in place until cell division, ensuring that each daughter cell receives a copy. To test this model directly in live cells, we used RFP labeled lacI to visualize lacO sites present on a plasmid containing a functional R1 Par operon and GFP labeled ParM to visualize the filaments directly at the same time. We find that ParM filaments are dynamically unstable in vivo and that the majority of filaments undergo assembly and rapid disassembly in less than a minute. In addition, the filaments appear to orient themselves along the long axis of a bacterium by running into the sides of the cell and then following them to the poles. The most striking result, however, is that the plasmids undergo continual and very rapid pole-to-pole movements even when not actively dividing. Our results indicate that plasmid partitioning by ParM is a very dynamic process and suggest a new model for polymer-based plasmid segregation.

924

**Functional Mapping of Arp2/3 Complex and NPF Residues Involved in Complex Activation**

A. D. Siripala, E. D. Goley, E. Znameroski, M. D. Welch; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

The Arp2/3 complex integrates binding of several different activating factors to promote actin nucleation and filament branching. Arp2/3 complex activity requires binding to actin and nucleation-promoting factors (NPFs), but the precise molecular interactions involved in complex remain unclear. To identify sites of interaction with binding partners and gain insight into the molecular mechanism of Arp2/3 complex activation, we have performed site-directed mutagenesis on conserved Arp2/3 complex surface residues. We have identified several mutations in putative NPF binding

regions of Arp2 and Arp3 that reduce nucleation activity of the complex. Additionally, we have generated a series of mutations in the Arp2/3 binding domain of the NPF WASP to identify complimentary residues critical for Arp2/3 binding and activation. Those Arp2/3 and WASP mutations found to abrogate nucleation activity are being tested for their effects on binding affinity using fluorescence anisotropy. We will then compare the effect of these mutations on NPF affinity with their effects on Arp2/3 conformation, measured using fluorescence resonance energy transfer (FRET). By this method we can determine whether NPF binding, conformational change, and activation are mediated by the same residues, or whether they are separable activities. To address the role of mother filament binding in Arp2/3 complex activity, we have generated mutations in surface residues of the p34 and p20 subunits, and have found that they cause a range of defects in nucleation. At least one of these mutations causes a decrease in F-actin binding. By developing a more detailed picture of the molecular interactions and conformational changes required for Arp2/3 activation, we hope to better understand the complex process by which multiple factors come together to promote regulated actin polymerization in the cell.

925

**Identification and Biochemical Characterization of Small Molecule Arp2/3 Complex Inhibitors**

N. Tomasevic, A. Russell, D. Pierce, Z. Jia, R. Sakowicz; Cytokinetics Inc., South San Francisco, CA

Dynamic rearrangements of the actin cytoskeleton underpin a variety of essential cellular processes like migration, polarization or endocytosis and are also used by cellular pathogens (e.g., Listeria). A key component of the cellular actin polymerization machinery is the seven subunit Arp2/3 complex, responsible for nucleation and branching of nascent actin filaments. We devised an actin-polymerization based high throughput screen for modulators of Arp2/3 complex activity, hoping to uncover pharmacological tools that would further understanding of the many physiological functions of the Arp2/3 complex. The screen found two distinct classes of Arp2/3 complex inhibitors represented by compounds CK-0944636 and CK-0993548. Both compounds are potent (<10uM) and specific Arp2/3 complex inhibitors, as observed in either WASP or ActA stimulated actin polymerization assays. Neither compound inhibited spontaneous or formin-nucleated actin polymerization at concentrations up to 100uM. Exposure to CK-0944636 significantly reduced the total amount of Arp2/3 nucleated actin polymer, producing sparse but highly branched actin structures. In contrast, the presence of CK-0993548 did not significantly affect the total amount of actin filament produced, but the resulting filaments were primarily linear with minimal branching. Both compounds inhibited Arp2/3 complex dependent cellular processes such as podosome formation and Listeria motility. Availability of specific Arp2/3 complex inhibitors provides the cell biology community with important tools for studying the dynamics and physiology of the actin cytoskeleton.

926

**Mechanism of Actin Nucleation and Branching by Arp2/3 Complex and Coronin**

K. M. Daugherty, M. Gandhi, B. L. Goode; Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA

The mechanism by which Arp2/3 complex nucleates and branches actin filaments remains poorly understood. When activated by WASp or other proteins, the Arp2/3 complex can nucleate new (daughter) filaments from the sides of existing (mother) filaments to form branched structures. However, it is unclear whether filament side binding and branching is a requisite for nucleation or instead represents only one form of Arp2/3-dependent nucleation. Further, it is unclear whether branched nucleation by Arp2/3 complex is essential *in vivo* and/or what other cellular factors might regulate branching. To address these issues, we targeted for mutagenesis the essential *S. cerevisiae ARC35* gene, which encodes the ARPC2/p35 subunit of Arp2/3 complex. Previous studies suggest that this subunit plays an important role in both filament side binding and transducing WASp-stimulated conformational changes to the complex. We integrated alanine substitutions at every conserved solvent exposed residue on p35 and identified surfaces essential for *in vivo* function. We also developed methods for purifying Arp2/3 complex from strains expressing these lethal alleles to assess their biochemical defects in nucleation and branching. We then used these mutants to map the coronin functional interaction surface on p35. Coronin interacts with p35 through its coiled-coil domain and is the only known direct inhibitor of Arp2/3 complex. Coronin stabilizes Arp2/3 complex in an open inactive conformation until filaments are present, which relieves inhibition. In this manner, coronin is thought to spatially restrict Arp2/3-mediated nucleation to the sides of filaments to promote branching. We identified one surface on p35 that suppresses *CRN1* overexpression defects *in vivo* and suppresses coronin inhibitory effects on Arp2/3 complex *in vitro*. The proximity of this surface to essential surfaces that may mediate filament side binding suggests a mechanism by which coronin regulates Arp2/3 activity.

927

**Investigating Arp2/3 Complex and Formins as Assembly Factors for Actin-based Surface Protrusions in Lymphocytes**

S. M. Nicholson-Dykstra, J. Lee, F. Li, H. N. Higgs; Biochemistry, Dartmouth Medical School, Hanover, NH

INTRO: The lymphocyte cell surface is not smooth, but is covered by actin-filament based protrusions. Primary blood lymphocytes possess short microvilli (0.3-0.4 microns long), whereas cultured leukemia or lymphoma lines often exhibit both ruffles and microvilli. Microvilli are hypothesized to play roles in surface receptor segregation which is necessary for extravasation (transit from blood to periphery). OBJECTIVE: Our objective is to determine the mechanisms of microvillar and ruffle assembly in lymphocytes. Two attractive assembly factors are Arp2/3 complex and the formin family proteins. METHODS: Utilize “dominant-negative” inhibition and RNAi-based protein suppression to determine roles of actin nucleators in surface protrusion formation. Additionally, utilize antibodies to determine protein localization and abundance. RESULTS: Our current evidence using a function-blocking construct (GFP-fusion of N-WASP CA region) suggests that Arp2/3 complex is not required for microvillar assembly in lymphocytes. We have identified seven formins in lymphocytes that exhibit appreciable mRNA levels, four of which also exhibit appreciable protein quantities. Currently, we are analyzing protein localization and functional significance of these formins. CONCLUSIONS: 1) Lymphocyte microvilli assemble through an Arp2/3 complex-independent mechanism; 2) A subset of formins is likely to act in surface protrusion assembly in un-activated lymphocytes.

928

**Pure Native WAVE2 Complex Is Tightly Inhibited and Cannot be Activated *In Vitro* by Rac or Nck**

A. M. Lebensohn, M. W. Kirschner; Department of Systems Biology, Harvard Medical School, Boston, MA

WAVE proteins are central regulators of Arp2/3 mediated actin assembly downstream of the small GTPase Rac. In cells WAVE forms a tight

heteropentameric complex, which plays a crucial role in regulating WAVE function. Two conflicting models describing the details of this regulation have been proposed. One model, based on *in vitro* experiments with native WAVE1 complex, poses that the complex is basally inhibited but can be activated by Rac and/or Nck. The second model, based on *in vivo* localization experiments and *in vitro* assays with recombinant reconstituted WAVE complexes, argues that the complex is basally active and that binding to activated Rac only serves to localize the active complex to sites of actin assembly. Based on *in vitro* studies with native WAVE2 complex we present findings at odds with both models. We found that pure native WAVE2 complex is basally inactive in actin polymerization assays containing Arp2/3 and actin. In fact the complex is so tightly inhibited that not even 1% of its expected activity could be detected in these assays. Unexpectedly, activated Rac failed to activate native WAVE2 complex even at extremely high concentrations. Importantly, Rac bound the native complex, including the WAVE2 subunit, in a nucleotide dependent manner. Nck also failed to activate the native WAVE2 complex when added alone or in combination with Rac. Finally, preheating the native WAVE2 complex to 57°C for 10 minutes before diluting it into the assay resulted in strong Arp2/3 dependent actin nucleation. Thus, native WAVE2 complex is tightly inhibited in its basal state but can be activated *in vitro*, albeit by extreme unphysiological conditions, to promote actin assembly. The physiological mechanism of WAVE complex activation remains unknown.

930

**The Nck-Interacting Kinase Increases Actin Nucleation Rates by Direct Phosphorylation of the Arp2/3 complex**

L. L. LeClaire,[1] M. Baumgartner,[2] R. D. Mullins,[3] D. L. Barber[1]; [1]Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA, [2]Institut of Medical Virology, University of Zurich, Gloriastr, Switzerland, [3]Cell and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA

The Arp2/3 complex is the major nucleator of actin filaments. By regulating the nucleation activity of the Arp2/3 complex, cells maintain precise control over actin polymerization and cytoskeleton assembly. The primary regulators of Arp2/3 complex nucleation activity are nucleation promoting factors of the WASP/Scar protein family. We propose, however, that phosphorylation is an additional mechanism for regulating activity the Arp2/3 complex. In MTLn3 rat epithelial carcinoma cells epidermal growth factor induces a rapid increase in phosphorylation of the Arp2/3 complex within 30 seconds, corresponding with an increase in total cellular F-actin. In vitro, phosphorylation of the Arp2/3 complex by Nck-interacting kinase (NIK) increases actin nucleation rates by 30%. In addition, NIK binds the Arp2/3 complex, and we identified the Arp2/3 binding site between amino acids 360 and 500 of the NIK regulatory domain. By using mass spectrometry we identified two phosphorylated threonine residues in the Arp2 subunit. These residues are located on subdomain 4 of Arp2 and are directly adjacent to arginine residues on the p20 subunit. These threonine and arginine residues are conserved in amino acid sequences from yeast, mice, and humans. By examining structural models of the Arp2/3 complex, we predict a model whereby phosphorylation of the Arp2 subunit facilitates a conformational change with the p20/p35 subunits. A phosphorylation-induced conformational change could enhance Arp2/3 activation by aligning the Arp2 and Arp3 subunits to form a nucleus for a new actin filament, thus providing a previously unrecognized mechanism for Arp2/3 complex regulation. Supported by NIH grants T32 DE07204 to LLL and GM47413 to DLB.

931

**Investigation of the Role of Nucleotide Binding to Arp2/3 Complex using X-ray Crystallography and Molecular Dynamics Simulations**

B. J. Nolen,[1] R. Littlefield,[2] P. Dalhaimer,[1] H. Chen,[1] T. Pollard[1]; [1]Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, [2]The Scripps Research Institute, La Jolla, CA

Arp2/3 complex stimulates formation of actin filaments at the leading edge of motile cells. The activity of the complex requires binding of ATP by both actin-related subunits in the complex, Arp2 and Arp3, and hydrolysis of ATP by Arp2. We have solved crystal structures of Arp2/3 complex with ATP or ADP both by soaking the nucleotides into preformed crystals and by co-crystallization. Co-complexes obtained through either method have nearly identical structures. In both cases, the nucleotides are immobilized on the face of subdomains 3 and 4 of Arp2, while subdomains 1 and 2 are flexible and absent from the electron density maps, as in the apoenzyme structure. This flexibility may explain why Arp2 does not hydrolyze ATP until the complex is activated. We have recently found that treating crystals of Arp2/3 complex with the crosslinking reagent gluteraldehyde decreases flexibility in the disordered regions of Arp2 and has allowed us to build additional regions of subdomain 1. In either soaked or co-crystallized complexes, ATP stabilizes a relatively closed conformation of Arp3 with the γ-phosphate bridging loops from opposite sides of the cleft. ADP-soaked crystals show that the nucleotide binds Arp3 in a unique conformation which favors an open cleft, revealing a conformational change that may occur in actin and Arps when the γ-phosphate dissociates from the ADP-Pi intermediate. Surprisingly, when ADP is co-crystallized with the complex, Arp3 has a closed cleft and the nucleotide does not exhibit an unusual conformation. We are currently using molecular dynamics simulations to investigate the relationship between nucleotide binding state and conformation in both Arp3 and actin.

932

**The Function of Arp2/3 Complex ATP Hydrolysis**

A. C. Martin, M. Welch, D. G. Drubin; MCB, UC Berkeley, Berkeley, CA

The Arp2/3 complex has been shown to nucleate actin filaments at the leading edge of motile cells and at endocytic sites. However, how the Arp2/3 complex at dendritic branches is released from membrane-bound nucleation promoting factors and how these dendritic branches are disassembled is not fully understood. Given the importance of ATP hydrolysis in actin turnover and the fact that Arp2 ATP hydrolysis occurs after nucleation, ATP hydrolysis by the Arp2/3 complex could regulate these events. Here, we identify an Arp2 mutant in *S. cerevisiae* that is unable to hydrolyze ATP upon nucleation. This mutant shows slightly enhanced nucleation activity *in vitro*, demonstrating that Arp2 ATP hydrolysis is not required for actin nucleation or branch formation. In contrast to other mutations we have identified that elevate Arp2/3 complex nucleation activity, this mutant exhibits severe defects in actin patch/endocytic internalization, suggesting a role for Arp2 ATP hydrolysis in regulating the structure of actin networks. Interestingly, the growth phenotype of this Arp2 hydrolysis mutant is specifically suppressed by loss-of-function mutations in activators of the Arp2/3 complex, suggesting that Arp2 ATP hydrolysis might regulate activator binding. In addition, actin patch disassembly is inhibited in this mutant, suggesting that Arp2 ATP hydrolysis may also regulate the turnover of filamentous actin networks. Interestingly, an equivalent mutation in Arp3 is synthetic lethal with the Arp2 hydrolysis mutant, suggesting that Arp3 ATP hydrolysis may also regulate Arp2/3 complex turnover *in vivo*. These results, along with further biochemical characterization of these mutants, will provide insights into the regulation

of the Arp2/3 complex.

933

**Role of p78/83 and Arp2/3 Complex in Baculovirus Pathogenesis**

E. D. Goley,[1] T. Ohkawa,[2] J. A. D'Alessio,[1] L. E. Volkman,[2] M. D. Welch[1]; [1]Molecular and Cell Biology, University of California-Berkeley, Berkeley, CA, [2]Plant and Microbial Biology, University of California-Berkeley, Berkeley, CA

Of the many pathogens that exploit their host cell's actin cytoskeleton during infection, baculoviruses are remarkable for the unique actin rearrangements they require for replication. Early in infection, baculovirus nucleocapsids induce actin cable formation in the cytoplasm of the host cell, a process hypothesized to mediate efficient translocation to the nucleus, the site of viral replication. Later, baculoviruses induce the polymerization of actin within the nuclei of infected cells, an apparently novel phenomenon that is essential for nucleocapsid assembly. Despite the critical nature of these manipulations of host actin, the molecular mechanisms underlying these changes and the function of actin in progeny production remain unresolved. To investigate the role of actin dynamics during baculovirus infection, we treated cells infected with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) with drugs that inhibit actin polymerization or depolymerization and demonstrate that these treatments prevent replication. At the molecular level, we find that an essential and conserved minor capsid protein, p78/83, bears significant homology to the Wiskott-Aldrich Syndrome protein family. We show that p78/83 is a robust nucleation-promoting factor for host Arp2/3 complex, suggesting a mechanism for nucleation of cytoplasmic actin cables that may mediate motility early in infection. Intriguingly, we find that p78/83 and Arp2/3 complex also colocalize with actin filaments in the nucleus later in infection, implicating these factors in the functionally critical nuclear polymerization of actin. Accordingly, mutation of conserved residues in p78/83 causes defects in its ability to activate Arp2/3 complex in vitro. AcMNPV bearing these same mutations are reduced in progeny production, with the worst mutants being unable to sustain virus viability. These data suggest that p78/83 mediates essential actin rearrangements during baculovirus infection by activating host Arp2/3 complex both in the cytoplasm and, strikingly, within the nucleus late in infection.

934

**Microrheology and Stress Fluctuation in Active Gels and Mechanosensitive Cells**

D. Mizuno,[1] R. G. Bacabac,[1] G. Koenderlink,[2] M. Atakhorrami,[1] F. MacKintosh,[1] T. H. Smit,[1] J. Klein-Nulend,[1] C. Schmidt[1]; [1]Vrije University, Amsterdam, The Netherlands, [2]Harvard University, Cambridge, MA

Active processes in cells such as crawling, intracellular transport, and mechanochemial transduction are in many cases related to the function of cytoskeletal networks composed of actin-myosin stress fibers or microtubules, and their associated proteins including motors. Non-thermal stress fluctuation caused by the motor proteins is one of the driving forces of these active processes. Rheological property of the cell (given by cytoskeletal networks) is also a key factor for determining the efficiency or speed of those processes. One intriguing aspect in elucidating cell activity is that the stress fluctuation and the rheological property might be influenced each other. Therefore, in this study, both stress fluctuation and local rheological property was measured simultaneously in mechano-sensitive cells(osteocytes) and in its in-vitro model of actin-myosin gel with ATP. Recently developed techniques called micro-rheology enables us to measure stress fluctuation and local rheological property of biological tissues. Two general approaches in microrheology pertain to measurement of probe-particle displacements embedded in the material investigated. The approach is either by inducing external forces on the probes (active mode) or by monitering the thermal and non-thermal fluctuations of the probes (passive mode). In equilibrium such as actin solution without myosin, active and passive modes gave the same results. However, out of equilibrium, extra stress fluctuation was observed at frequencies lower than 100Hz in both osteocytes and actin-myosin gel. Results were: (1) At 37°C, stress fluctuation of cell has $\omega^{-2}$ frequency dependence. (2) At 20°C, cell loses process with faster fluctuation and hardens its body. (3) At high ATP, non-processive myosin cannot give significant stress fluctuation to actin network. (4) At low ATP, myosin filaments works cooperatively and gives significant stress fluctuation. (5) Non-thermal stress fluctuation can be suppressed by external potential larger than matrix viscoelasticity.

935

**Theoretical Study of Force Generation and Kinetics of Actin Filament Bundles**

D. Vavylonis,[1,2] B. O'Shaughnessy[1]; [1]Chemical Engineering, Columbia University, New York, NY, [2]Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT

Many types of cellular processes are driven by the polymerization and depolymerization of actin filament bundles growing or shrinking against cellular loads. The Brownian ratchet is a mechanistic mathematical model which describes force generation by polymerizing filaments. We used numerical and analytical methods to study the dynamical and equilibrium properties of the Brownian ratchet generalized to the case of stiff actin bundles. For an equilibrium polymerization process (ignoring hydrolysis of ATP bound to actin subunits) the bundle stall force depends linearly on the number of filaments and logarithmically on actin monomer concentration, $c$ (Hill and Kirschner, *Int. Rev. Cytol.* **78**, 1, 1982). The magnitude of time averaged bundle length and force fluctuations at stall is independent of $c$, but we find that the time dependence is strongly dependent on $c$. For high stall forces (high $c$) most filament tips are crowded very close to the load and the collective bundle kinetics are exponentially slow. For small stall forces ($c$ close to $c_{crit}$), the filament length distribution becomes broad leading to fast relaxation. We accounted for ATP hydrolysis by actin using the accepted picture of fast hydrolysis after polymerization followed by slow release of phosphate. We find that the main effects of hydrolysis with respect to equilibrium are (i) a slight change in the stall force and (ii) accelerated dynamics near $c_{crit}$. Formins associated with the filament ends can modify the bundle dynamics. We consider possible mechanisms by which formins can promote fast bundle response even close to stall and high $c$, conditions under which formin-free bundles would elongate slowly. This could be a biologically relevant process since formins localize at the tip of actin bundles in filopodial protrusions.

936

**In Silico Modeling of Actin Based Motility: Bugs, Beads, Vesicles, and Cell Motility**

J. B. Alberts, G. M. Odell; Center for Cell Dynamics, University of Washington, Friday Harbor, WA

We believe that fully understanding the complex (emergent) behavior of many cellular processes requires mathematical modeling/simulation of the

small-scale biochemical and mechanical details. Such a modeling approach is necessary because the source of the complexity is not often obvious; the dynamics derive from the interplay of thousands of simple interactions with stochastic influences. We thus construct detailed biochemical/mechanical models that are, in essence, computational formalizations of a reductionist view of cellular processes. One example, our "in silico reconstitution" of the motility of the bacteria Listeria monocytogenes is described in Alberts JB & Odell GM, PLoS Biology 2(12). We continue to refine and modify this model, and here apply it to the motility of beads and deformable vesicles. We specifically address the force-velocity relationship, and the effect of geometry on force-generation, for dendritic actin network based motility. In addition we present, as a learning tool, a freely downloadable Java applet that allows for the interactive 3-dimensional exploration of a growing and branching actin network. The user of this program can vary the concentrations proteins (e.g. actin monomer, capping protein, Arp2/3), as well as change the nature of several biochemical interactions, in order to explore the consequence on actin network morphology (e.g. branching frequency, network density).

937

**Mechanical Properties and Force Generation of Actin Networks near the Polymerization Transition**

A. Pomerance, W. Losert; Institute for Research in Electronics and Applied Physics, University of Maryland, College Park, MD

Gradients in the concentration of actin filaments and in Actin Binding Proteins (ABP) are characteristic of the leading edge of moving cells. Similarly, forces of several pN are characteristically exerted at the leading edge. We present studies of the mechanical properties of actin networks with focus on gradients in network properties and on the response to large forces. In the presence of 2 mM $Mg^{2+}$ and 50 mM $K^{+}$, the critical concentration (CC) for polymerization of actin is 0.03 mg/mL, but in the absence of these ions the CC increases by two orders of magnitude. By studying concentrated actin samples (approximately 3.5 mg/mL) with and without added ions, we compare the behavior near and far above the CC. To study the response to large forces we use holographic laser tweezers to pull microspheres through an actin network. We have found that actin networks far above the CC strongly resist pulling, and have a well-defined relaxation time. Near the CC, beads are easily pulled through the actin networks, and the relaxation is far more variable, which indicates that the actin filaments may be more dynamic and breakable. We generate gradients in actin network structure using a thermal gradient. We observe that in actin networks near the CC, embedded beads are pushed towards the cold side (weaker network) at approximately 0.1 microns/second. This indicates that the flux of energy associated with the thermal gradient may generate forces in a partially polymerized filament network. Gradients in chemical potential, e.g. due to gradients in ABP may also lead to force generation. To study this, we have developed microfluidic devices that maintain a stable, quasi-static gradient in large macromolecules for roughly one hour. Work supported by NIH grant R21BE00328501

938

**Models and Simulations of Actin Dynamics in Graded Polarity (GP) Bundles**

R. S. Littlefield,[1] C. M. Waterman-Storer,[2] G. M. Odell[1]; [1]Center for Cell Dynamics, Friday Harbor Labs, University of Washington, Friday Harbor, WA, [2]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

Although contractile actin bundles are important for cell movement, adhesion, and division, it is unknown how actin filament organization is generated during bundle assembly and maintained while they contract. In graded polarity (GP) bundles, the average polarity of actin filaments changes continuously along their length with all barbed ends pointing toward the ends of the bundle, i.e. opposite directions at opposite ends. Using live cell fluorescent speckle microscopy and mathematical models, we investigated the dynamics of actin filaments during the assembly and contraction of GP bundles. De novo GP bundle assembly occurs from the lateral collapse of the lamellar meshwork on the dorsal surface at the lamella-cell body boundary. Actin speckles along GP bundles converge towards each other at a rate of 0.04 - 0.08 percent per minute, but the speckles do not cross each other in kymographs. In contrast, Monte Carlo simulations of GP bundles containing sliding actin filaments predict that speckle tracks would cross. This conflict suggests that actin filaments are dynamically rebuilding the bundle as it contracts to reproduce the speckle behaviors we observe. Indeed, even though the total actin fluorescent intensity remained very stable and uniform along GP bundles, we observed actin speckles flow inward from the ends of GP bundles as well as speckle appearance and disappearance in the middle of GP bundles. We are currently modifying our Monte Carlo simulations to incorporate these observations and determine whether actin filament dynamics can account for GP bundle speckle dynamics. We will also present results from individual-based models which explore how myosin-based contraction and crosslinks by alpha-actinin (which we have observed experimentally) contribute to the movement and dynamics of actin filaments within the GP bundles to produce a stable organization that is able to generate force.

## Conventional Myosin (939-956)

939

**Mts1 (S100A4) Regulates Cellular Motility via a Direct Interaction with Myosin-IIA**

Z. Li, A. R. Bresnick; Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY

Mts1, a member of the $Ca^{2+}$-regulated S100 family of proteins, has been characterized as a metastasis factor and is thought to regulate the motility and invasiveness of cancer cells. Previously, we demonstrated that mts1 specifically binds to nonmuscle myosin-IIA on residues 1909-1924 of the heavy chain, and inhibits the assembly of myosin-IIA monomers into filaments and promotes the disassembly of pre-existing myosin-IIA filaments in a calcium-dependent manner. To examine how the biochemical properties of mts1 impact cell motility, we determined how the expression of mts1 affected protrusive behavior during chemoattractant-stimulated motility. In addition, we prepared an antibody to the mts1 binding site on the myosin-IIA heavy chain that has comparable effects on myosin-IIA assembly and motor activity as mts1. Our studies demonstrate that mts1 modulates cellular motility by effecting the orientation and localization of protrusions, with mts1 expressing cells displaying few side protrusions and extensive forward protrusions during chemotaxis as compared to control cells. Microinjection experiments demonstrate that the antibody elicits the same effects on protrusive activity as mts1. These results establish mts1 as a critical regulator of myosin-II mediated motility and suggest that the regulation of myosin-IIA filament assembly is an important regulatory control for cellular motility.

940

**Distinct Roles of Nonmuscle Myosin II Isoforms in The Regulation of MDA-MB 231 Breast Cancer Cell Spreading and Migration**

V. Betapudi, L. Licate, T. T. Egelhoff; Physiology and Biophysics, R# E625A, Case Western Reserve University, Cleveland, OH

Nonmuscle myosin II is an important component of actomyosin cytoskeleton that is believed to play a key role in cell migration in many settings, including cancer metastasis, atherosclerosis, rheumatoid arthritis, wound healing, and angiogenesis. During migration protrusion events at spreading lamellipodia are widely regarded as being driven by F-actin polymerization independent of myosin II, with cell body translocation and posterior contraction generally believed to be myosin II-dependent events. However, the exact mechanical roles of myosin II during cell migration remain remarkably poorly understood. In the present study, we show a transient increase in RLC phosphorylation that correlates the recruitment of myosin IIA and IIB isoforms into distinct periodic rows in the lamellipodia during initial phases of spreading on fibronectin. However, Rho kinase inhibition by using Y-27632 blocks myosin IIA recruitment to lamellipodia with a modest affect on spreading. On the other hand, inhibition of MLCK by using ML-7 dramatically reduced spreading. Depletion of myosin IIA via siRNA impaired migration, but enhanced lamellar spreading, while depletion of myosin IIB impaired not only migration, but also impaired initial rates of lamellar spreading. These results indicate that both isoforms are critical for the mechanics of cell migration, with myosin-IIB appearing to have a specific role in the mechanics of lamellar protrusion events.

941

**Role of Myosin II in Regulating Keratocyte Path Persistence**

E. L. Barnhart, J. A. Theriot; Biochemistry, Stanford University, Stanford, CA

Rapidly locomoting epithelial fish keratocytes display remarkable path persistence, migrating in straight lines even in the absence of any external directional cues. Tracking the cell body centroid of moving keratocytes shows that the cell body oscillates a few microns from side-to-side along a straight path. The regularity of this lateral oscillation suggests that path persistence is maintained by coordination of the rate of protrusion and retraction between the left and right sides of the cell such that any deviation from a straight path is overcorrected. To determine whether lateral oscillation is periodic, we tracked a large population of cells, fit each cell track to a smooth curve, and performed Fourier analysis on the distance between the fit line and the track. All cells exhibited significant periodicity, with periods ranging from 20 to 70 seconds, and there was a strong negative correlation between the period and cell speed. We are presently investigating the mechanism governing this periodic oscillation. To determine whether myosin II contraction at the trailing edge of migrating keratocytes contributes to the oscillation, we transfected cells with a YFP-myosin II regulatory light chain transgene. Live cell imaging of locomoting keratocytes showed that YFP-MRLC accumulates in two pronounced spots on either side of the cell body along the rear edge of the cell. We measured the fluorescent intensity in each of these myosin II rich spots over time and found that they increased in intensity in an anticorrelated periodic fashion. The difference in intensity between the left and right spots was strongly correlated with the position of the cell body, suggesting that myosin II contributes to lateral oscillation. We are currently using a number of inhibitors of myosin II to further characterize its role in cell body oscillation and path persistence.

942

**Dynamic Redistribution of GFP-Myosin II and Traction Forces in Wild-type *Dictyostelium* Movement**

M. Lombardi, D. Knecht, J. Lee; Molecular and Cell Biology, University of Connecticut, Storrs, CT

Cell movement requires the generation of a protrusive force at the leading edge and contractile forces at the cell rear to facilitate retraction. The transmission of contractile forces to the external substratum, via cell adhesions, results in the production of traction forces which “pull” the cell body forward and facilitates retraction at the rear. In our previous studies we investigated traction forces generated by wild-type and myosin II null mutant *Dictyostelium discoideum* cells using a gelatin traction force assay. Our data showed that wild-type cells produced traction forces ten times greater than the myosin II null cells, however the null cells are still capable of generating small traction forces independently of myosin II. In wild-type cells we find that the highest traction forces are located at the rear, while the high traction forces in the null cells are randomly distributed. Another observation in our data is the development of high traction forces just behind the leading edge of a moving wild-type cell that resembled a ‘contractile collar’. To investigate the relationship between traction force production and the location of myosin II we used a GFP-myosin II expressing *Dictyostelium* and the gelatin traction force assay. We observed that GFP-myosin II redistribution does not directly correlate with high traction forces generated by a moving cell. Surprisingly high traction forces at the rear of the cell are shown prior to highly observable aggregated GFP-myosin II. We find that after retraction, traction forces lower while the fluorescence intensity of GFP-myosin II increases. We have also unexpectedly observed that GFP-myosin II is not found in the ‘contractile collar’. Our data suggests that the generation of high traction forces precedes an observable increase in the aggregation of GFP-myosin II in moving *Dictyostelium*.

943

**FRET Analysis Comparing Myosin II Isoforms in Contracting A7r5 Smooth Muscle Cells**

J. E. Black, G. L. Wright; Physiology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV

Recent evidence has indicated that contraction of A7r5 cells is accompanied by a significant loss in actin stress fiber structure with dissociation of actin and myosin. In the present study we investigated the interaction of the principal isoforms of myosin II (Sm1 and Sm2) with two isoforms of actin, α-actin and β-actin. Whole cell fluorescence resonance energy transfer (FRET) analysis was utilized as an index of the degree of association in control and phorbol-12,13-dibutyrate (PDBu) contracted cells. Average efficiencies indicating association of Sm1 with α-actin were 17 ± 2% in control cells, 10 ± 1% ($p=0.039$) in 0.1μM phorbol contracted cells, and 4 ± 0.9% ($p<0.001$) in 10μM phorbol indicating dissociation of α-actin and this myosin isoform in contracted cells. Similarly, association of Sm1 with β-actin was 18 ± 2%, 12 ± 2%, 5 ± 0.6% ($p<0.0001$), respectively. In marked contrast, there were no significant changes in efficiencies seen when the Sm2 isoform was compared with α-actin or β-actin. This data suggest that the association of Sm1 with actin is altered during contraction; whereas that of Sm2 is constant. We speculate that Sm1 is important in the contraction mechanism while Sm2 is important in maintaining structural integrity role throughout the contraction.

944

**Development of a Mts1 (S100A4) Biosensor to Track Activation In Vivo**

S. C. Garrett,[1] D. Gremyachinskiy,[2] K. M. Hahn,[2] A. R. Bresnick[1]; [1]Biochemistry, Albert Einstein College of Medicine, Bronx, NY, [2]Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Myosin-II is highly dynamic as cells undergo directed motility with filament assemblies occurring primarily in lamellae, in the perinuclear region and at the trailing edge. Mts1, a member of the S100 family of proteins, binds specifically to nonmuscle myosin-IIA in a calcium-dependent manner and regulates the monomer-polymer equilibrium of myosin-IIA filaments. To obtain novel information regarding the spatial and temporal activation of mts1 during directed cell motility we developed an mts1 biosensor to report transient or localized calcium binding in vivo. This unique reagent will allow the temporal and spatial distribution of activated mts1 to be examined directly during the motility cycle, and will permit a direct correlation between localized, transient activation of mts1 and the regulation of myosin-IIA assemblies during directed motility. The biosensor was constructed using the novel merocyanine dye ISO-IAA, which displays sensitivity to both solvent polarity and hydrogen bonding, thus small changes in protein conformation can strongly influence the fluorescence properties of the dye. Similar to other S100 proteins, mts1 undergoes a significant conformational change upon calcium binding to the C-terminal EF-hand in which helix 3 extends outward exposing a hydrophobic cleft that forms the target binding surface. The biosensor was generated by the derivatization of a single cysteine residue (C81), proximal to the hydrophobic pocket, with ISO-IAA via a reactive iodoacetamide group. The resulting biosensor displays a 2.4-fold increase in fluorescence intensity upon calcium binding and myosin-IIA binding does not significantly affect the fluorescence intensity of the biosensor. Biochemical analyses demonstrate that the mts1 biosensor has the same binding affinity for calcium and myosin-IIA as the wild-type mts1. Furthermore, in 3T3 fibroblasts the biosensor shows diffuse fluorescence with no sign of aggregation. In vivo studies examining mts1 activation are in progress.

945

**Myosin II Dynamics in *Dictyostelium* and Mammalian Cells Assessed via GFP-Myosin II Fusions and Fluorescent Recovery After Photobleaching (FRAP)**

M. T. Breckenridge,[1] S. Yumura,[2] M. Yoshida,[2] V. Betapudi,[1] L. S. Licate,[1] Y. Iwadate,[2] A. Nagasaki,[3] T. Q. P. Uyeda,[3] T. T. Egelhoff[1]; [1]Dept Physiol Biophys, Case Western Res Sch Med, Cleveland, OH, [2]Dept Biology, Yamaguchi University, Yamaguchi, Japan, [3]Gene Function Research Center, National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan

Nonmuscle myosin II is a critical player in many cell motility processes, including cell migration, cytokinesis and vesicle trafficking. In vivo this protein exists in a highly dynamic equilibrium between a cytosolic monomer pool and assembled bipolar filaments that associate with the cytoskeleton to produce mechanical force. Mechanisms controlling filament assembly and subcellular localization in vivo remain poorly understood. We present here parallel studies performed with GFP-myosin II fusions in the amoeba Dictyostelium and in human breast cancer cells demonstrating that FRAP analysis can be used to quantify assembly behavior in vivo and to identify mechanisms and upstream kinases regulating assembly in vivo. In Dictyostelium, FRAP analysis performed in candidate myosin heavy chain kinase (MHCK) gene disruption lines demonstrates that three enzymes, MHCK A, B, and C, play a predominant and critical role in regulating the equilibrium between the monomer pool and assembled filament, both in the contractile ring and during interphase. In mammalian cells we find that GFP-myosin IIA and IIB display remarkably rapid turnover between the cytosolic pool and assembled cytoskeletal material, both within the contractile ring and in extending lamellipodia of spreading cells ($t_{1/2}$ for recovery < 1 min). We further demonstrate that myosin IIA and IIB isoforms have distinct assembly/disassembly kinetics, suggesting possible distinct in vivo roles and regulation for these two ubiquitous myosin II isoforms. Further studies are in progress addressing mammalian myosin II isoform assembly regulation in the context of cell migration and spreading, and during cytokinesis.

946

**Reduction of β-Integrin Expression and Cell Attachments in Nonmucle Myosin II-B Null Mouse Embryonic Fibroblasts**

K. Kim, R. S. Adelstein; Lab. of Molecular Cardiology, NHLBI, NIH, Bethesda, MD

Previous work has shown that ablation of nonmuscle myosin heavy chain (NMHC) II-A in mice results in defects in cell adhesion and loss of E-cadherin and β-catenin localization to cell-cell adhesion sites (Conti et al. J. Biol. Chem. 279:41263, 2004). Here we study the function of NMHC II-B in mouse embryonic fibroblasts (MEFs), a cell line that normally contains myosin II-A and II-B, but not II-C. We compared a MEF that was derived from a mouse that had been ablated for NMHC II-B with a MEF derived from a wild-type mouse. The myosin II-B ablated MEFs showed a decrease in the content of both β1 and β3-integrins compared to wild-type using immunoblot and FACS analysis. There was no change in α5 and αv-integrins in these cells. Of note the II-B ablated MEFs showed a decreased attachment to a number of extracellular matrix proteins compared to wild-type MEFs. The defect in both β1-integrin content and cell attachment could be rescued by transfection of II-B ablated MEFs with GFP-II-B. When siRNA to NMHC II-B was used to lower the content of myosin II-B in wild-type MEFs there was a reduction in β1-integrin as well as a decrease in cell-matrix attachment in the siRNA-treated cells. Interestingly, MEFs that contain a mutant myosin II-B (a single amino acid mutation, Cys in place of Arg 709) that has a decreased actin-activated MgATPase activity and *in vitro* motility (Kim et al. J. Biol. Chem. 280:22769, 2005) do not show any abnormality in integrin content or matrix attachment. This implies that myosin motor activity may not be required for β-integrin expression. Our results suggest that NMHC II-B is involved in the attachment of MEFs to the extracellular matrix by regulating β-integrin expression.

947

**Transcriptional Response to Myosin Type II Deficiency in *Saccharomyces cerevisiae***

J. F. Rodriguez-Quinones, F. E. Rivera-Molina, D. Gomez-Garzon, J. R. Rodriguez-Medina; Biochemistry, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico

The *Saccharomyces cerevisiae MYO1* gene encodes myosin type II (Myo1p), a protein related to cytokinesis and cytoplasmic vesicle transport in higher eukaryotes. Myo1p deficiency in yeast (*myo1)* is characterized by altered cell division, cell enlargement, osmotic sensitivity and delocalized chitin deposition, suggesting that it may also be required during cell wall biogenesis. To identify the nature of the cell wall damage caused by

myosin type II deficiency, a transcriptional profile analysis of *myo1* strains was developed using yeast oligonucleotide microarrays. The hybridized arrays were analyzed using statistical methods considering a 2-fold expression change for the mRNA levels of 6,256 known ORFs in the yeast genome. Based on these specifications, 409 up- and 264 down- regulated genes were identified and categorized under cell wall organization and biogenesis, stress response, transport, metabolism, carbohydrate metabolism and protein biosynthesis functions. Certain genes that were up-regulated compared favorably with those previously identified by others in transcriptional analysis of cell wall-related mutations (*PIR3, SPI1, ECM13, ECM4, YGP1*), stress response (*HSP12, DDR2, and YML131W* ) and cell wall damage (*PIR3, HSP12, YHR097C*). Conversely, the reported up-regulation of *SLT2* transcription during transient and chronic cell wall damage was not observed. This study also revealed that despite a requirement for Chs3p expression in *myo1* strains, *CHS3* transcription was not up-regulated. We conclude that *myo1* strains induce a limited subset of genes regulated by the cell wall integrity pathway, but their overall transcriptional response is clearly different from previous studies. This can be attributed to differences in growth conditions used in our study or in the signaling pathways induced by Myo1p deficiency. This work was supported by PHS grants to the University of Puerto Rico, MBRS-SCORE (S06-GM08224) with partial support from RCMI (G12-RR03051) and MBRS-RISE (R25-GM61838).

948

**Conditional Ablation of Nonmuscle Myosin II-B in the Mouse Heart**

A. K. Singh,[1] Q. Wei,[1] K. Takeda,[2] P. Zerfas,[3] C. Birdsall,[1] S. Sidenko,[1] R. S. Adelstein[1]; [1]NHLBI, NIH, Bethesda, MD, [2]NIAID, NIH, Bethesda, MD, [3]OD/ORS, NIH, Bethesda, MD

Ubiquitous ablation of nonmuscle myosin heavy chain II-B (NMHC II-B) in mice resulted in defects in the heart and brain, leading to lethality between embryonic day (E)14.5-E18.5. To understand the physiological role of NMHC II-B in the adult mouse heart, we used a Cre/loxP strategy to ablate NMHC II-B specifically in the heart by generating mice with exon 2 of *Myh 10* flanked by loxP sites. These mice were then crossed with mice expressing Cre-recombinase under control of the α-cardiac MHC promoter, which is activated later in development than the NMHC II-B promoter. In contrast to conventional NMHC II-B ablated mice, 80% of the $B^{\alpha card}/B^{\alpha card}$ mice survived to become adults. These mice developed a novel phenotype beginning at 6 months. EKG abnormalities including bradycardia, right axis deviation and a posterior hemiblock were noted. By 10 months, the echocardiogram of these mice showed a decreased fractional shortening of the left ventricle (21% vs 47% for wild type mice) indicating a compromised cardiac function. Histopathological changes were seen by both light and EM microscopy in these hearts. At 6 months inflammatory cell infiltrates were in the interstitium of all four chambers. By 10 months changes included marked interstitial fibrosis, in which large areas of the myocardium were replaced with connective tissue. Previously, we had shown that NMHC II-B was present in the Z-line and intercalated disks (Takeda et al.,*Cell Motil. Cytoskel.* 2000: **46**, 59). Interestingly, both of these structures were found to be abnormal using EM. Most dramatic were the changes in the intercalated disks, which were markedly widened and distorted compared to wild-type controls. These results show that NMHC II-B, in addition to being required for normal cardiac development, also plays an important role in the adult mouse heart.

949

**Nonmuscle Myosin II-B is Required for Mouse Spinal Cord Development**

X. Ma, J. Bao, S. Kawamoto, C. Liu, R. S. Adelstein; Laboratory of Molecular Cardiology, National Institutes of Health, Bethesda, MD

Ablation of nonmuscle myosin heavy chain II-B (NMHC II-B) or expression of reduced amounts of R709C mutant NMHC II-B results in the development of hydrocephalus as early as embryonic day 13.5 (E13.5) in the mouse. Except for the abnormal protrusion of facial neurons into the developing fourth ventricle, no blockage of the cerebral ventricles is seen in these mice. In spite of this all of the cerebral ventricles are enlarged in the mutant mice compared to wild type mice. We therefore analyzed spinal cord development in various mutant mice including NMHC II-B knockout, R709C II-B mutant hypomorphic and non-hypomorphic mice, as well as mice expressing NMHC II-A in place of II-B. Disruption of the ventricular neuroepithelium was observed by E11.5 in both knockout and R709C mutant hypomorphic mice along the entire spinal cord, and neuroepithelial cells protruded into the developing central canal. Between E13.5- E14.5 the central canal was completely blocked. More severe abnormalities were seen in knockout mice compared to the R709C mutant hypomorphic mice. Interestingly, increased expression of mutant II-B to wild type levels in non-hypomorphic mutant mice prevented blockage of the central canal and development of hydrocephalus, although these mice still suffered from novel abnormalities that were not seen in knockout and hypomorphic mutant mice. When NMHC II-A is expressed in place of II-B, no disruption of the neuroepithelium of the central canal or hydrocephalus was found in NMHC II-B ablated mice. These results indicate that NMHC II-A can replace NMHC II-B in maintaining the integrity of the neuroepithelium in the central canal, although other defects in the brain and heart were not rescued. In conclusion, NMHC II-B plays an important role in maintaining the integrity of the neuroepithelium in the spinal cord.

950

**Transcription Factor Sp3 and DNA Methylation Regulate the Nonmuscle Myosin Heavy Chain II-C Promoter**

M. Chung, R. S. Adelstein, S. Kawamoto; NHLBI, National Institutes of Health, Bethesda, MD

We have previously identified two alternative promoters for the mouse nonmuscle myosin heavy chain (NMHC) II-C gene which are utilized in a tissue-dependent manner. Both promoters lack a TATA box and show a high GC content. The promoter located more distally from the common exon 2 is utilized in a wide variety of tissues in adult mice, with the exception of the lung where the more proximal promoter is predominantly utilized. However, many cell lines derived from these tissues express NMHC II-C mRNAs at very low levels, presumably reflecting the low expression of these mRNAs at early embryonic stages as well as their absence from embryonic stem cells. To explore the mechanisms underlying repression of the distal promoter, we first examined the DNA methylation status of the promoter and flanking regions. Results from analysis of genomic DNA by a methylation-sensitive restriction enzyme, treatment of cell lines with the nonmethylatable analog Aza-C, and analysis of reporter constructs methylated *in vitro* suggest a major contribution of CpG methylation to repression of the distal promoter, but not the proximal promoter. Furthermore, treatment of cell lines with the histone deacetylase inhibitor, trichostatin A (TSA), and to a greater extent with TSA and Aza-C together, leads to activation of the distal promoter activity. Chromatin immunoprecipitation assays, siRNA to Sp1 and Sp3 and overexpression of Sp1 and Sp3 demonstrate that the transcription factor Sp3, but not Sp1, participates in activation of the distal promoter when repression is relieved. These results suggest that DNA methylation and histone deacetylation play critical roles in repression of the distal promoter of the NMHCII-C gene and that Sp3 plays a role in activation of this promoter.

951

**Increased Expression of the Inserted Myosin II-C Isoform in Tumor Cell Lines**

S. S. Jana, R. S. Adelstein; Laboratory of Molecular Cardiology, National Institutes of Health, Bethesda, MD

A previous report from this laboratory revealed that the newly recognized nonmuscle myosin heavy chain II-C (NMHC II-C) contains an alternative exon composed of 24 nucleotides encoding 8 amino acids. This exon is spliced into loop *I* of NMHC II-C and is present in a wide variety of tissues and cell lines. Insertion of this exon leads to an increase in the actin-activated MgATPase activity and *in vitro* motility of heavy meromyosin II-C compared to the noninserted isoform (Kim et al (2005) *J. Biol. Chem.* 280, pp 22769-75). RT-PCR analysis reveals that the mRNA encoding the inserted isoform is highly expressed in many human epithelial tumor cell lines such as liver, HepG2; prostate, PC-3; pancreas, PANC-1; breast, MCF-7, and lung, A-549 cells. Interestingly, when we quantitated the expression level of all three nonmuscle myosin isoforms (NMHC II-A, II-B and II-C) in tumor cell lines vs normal cell lines, the expression of inserted II-C was elevated (>8 fold) in the tumor cell lines compared to the normal cell lines. In contrast NMHC II-A and II-B were decreased (>3 and >4 fold respectively) in the tumor cell lines as determined by scanning immunoblots. Analysis of breast, kidney and lung tumors from humans confirmed the increase in NMHC II-C expression in all three tumors compared to their normal associated tissue. Using siRNA to reduce the inserted isoform by 90% in the A549 lung epithelial cancer cell line leads to 80% growth inhibition at 156 h. This inhibition of growth was almost completely rescued by exogenous expression of the inserted NMHC II-C, but only partially rescued by noninserted II-C. These studies suggest a putative role for the NMHC II-C isoform in certain epithelial tumor cell lines.

952

**Myosin Heavy Chain Kinase A from *Dictyostelium* Possesses a Novel Actin Binding Domain that Cross-Links Actin Filaments**

M. Russ, O. Ali, K. Amber, P. Steimle; Biology, University of North Carolina at Greensboro, Greensboro, NC

Myosin heavy chain kinase A (MHCK-A) catalyzes the disassembly of myosin II filaments in *Dictyostelium discoideum* via phosphorylation of the "tail" region of the myosin heavy chain. MHCK-A is recruited to actin-rich cortical sites and is preferentially enriched at sites of pseudopod formation. MHCK-A possesses an amino-terminal "coiled-coil" domain (designated C1-498) that mediates the oligomerization, cellular localization, and actin binding activities of the kinase. We have shown that C1-498 binding to F-actin leads to a 40-fold increase in MHCK-A activity, and thus may represent a potent mechanism for achieving highly-localized and robust disassembly of myosin II filaments in the cell. In the current study, we examined the actin-binding characteristics of the "coiled-coil" domain as a means of gaining insight into the mechanisms by which F-actin-mediated activation of MHCK-A, and thus myosin II filament disassembly, can be regulated in the cell. Co-sedimentation assays revealed that C1-498 protein binds cooperatively to F-actin with an apparent $K_D$ of ~0.5 μM and a stoichiometry of ~5:1 (actin:C1-498). By contrast, assays of "coiled-coil" domain truncations lacking either amino-terminal (C121-498) or carboxy-terminal (C1-452) regions exhibited ~50% reductions in actin binding, and a combined truncation lacking both ends (C121-452) exhibits complete loss of actin binding activity. Studies examining the nature of the interaction with F-actin revealed that C1-498 has the ability to cross-link adjacent actin filaments into bundles. These results are particularly notable since they represent the first demonstration of a serine/theronine protein kinase possessing an actin cross-linking domain. In a larger context, our results suggest that changes in the accessibility of actin-binding determinants in the "coiled-coil" domain leads to localized cross-linking of actin filaments which, in turn, may activate MHCK-A-mediated disassembly of myosin II filaments at specific cell sites.

953

**A Novel Role for Myosin II in Insulin-Stimulated Glucose Uptake in 3T3-L1 Adipocytes**

P. Steimle, K. Fulcher, Y. Patel; Biology, University of North Carolina at Greensboro, Greensboro, NC

Insulin-stimulated glucose uptake requires the activation of several signaling pathways to mediate the translocation and fusion of GLUT4 vesicles from an intracellular pool to the plasma membrane. The studies presented here show that inhibition of myosin II activity impairs GLUT4-mediated glucose uptake but not GLUT4 translocation to the plasma membrane. We also show that adipocytes express both myosin IIA and IIB isoforms, and that myosin IIA is recruited to the plasma membrane upon insulin stimulation. Taken together, the data presented here represent the first demonstration that GLUT4-mediated glucose uptake is a myosin II-dependent process in adipocytes. Based on our findings, we hypothesize that myosin II is activated upon insulin stimulation and recruited to the cell cortex to facilitate GLUT4 fusion with the plasma membrane. The identification of myosin II as a key component of GLUT4-mediated glucose uptake represents an important advance in our understanding of the mechanisms regulating glucose homeostasis.

954

**PDGF-mediated Activation of PKC controls Myosin Filaments Disassembly**

S. Komatsu, M. Ikebe; Physiology, University of Massachusetts Medical School, Worcester, MA

Phosphorylation of the regulatory light chain of myosin II (MLC) may play an important role in cell motile processes through the rearrangement of actomyosin filaments by facilitating motor activity and myosin filament stability. In vitro biochemical studies have shown that the function of myosin II is regulated by phosphorylation of MLC by MLC kinases and protein kinase C (PKC). Phosphorylation of MLC by MLC kinases (Thr-18 and Ser-19) promotes its motor activity and filament formation. On the other hand, phosphorylation of MLC by PKC (Ser-1 and Ser-2 and Thr-9) decreases the binding affinity of myosin II for actin and the affinity of MLC for MLCK thus down regulating the actin-activated ATPase activity of myosin II. However, the physiological significance of this inhibitory phosphorylation has not been established. To address this question, we developed a site-specific phospho antibody (pS1 Ab) that recognizes phosphorylated MLC at the inhibitory sites but not the activation sites. Immunochemical analysis using pS1 Ab revealed that phosphorylation of the inhibitory sites is correlated with platelet-derived growth factor (PDGF)-induced cell migration. Interestingly, reorganization of actomyosin filaments in response to PDGF stimulation is associated with the increase of phosphorylation at the inhibitory sites. The phosphorylation of myosin II at the inhibitory sites in response to PDGF was attenuated by the specific PKC inhibitors (GF109203X and Go6976), but not PI3 kinase (LY294002) and MEK (PD98059) inhibitors. To further clarify the physiological role of phosphorylation of MLC at the inhibitory sites, unphosphorylatable MLC mutant (SS/AA-MLC) was introduced into NIH3T3 cells. Expression of SS/AA-MLC but not wild type MLC inhibited the PDGF-induced stress fiber disassembly. These results suggest that phosphorylation of MLC at the inhibitory sites by PKC plays an important role in dynamic reorganization of myosin during cell migration.

955

### The Cyclin Dependent Kinase 5 Activator p39 Binds Myosin Essential Light Chain

B. K. Tripathi, D. R. Ledee, P. S. Zelenka; Laboratory of Molecular and Devempmental Biology, National Eye Institute / National Institutes of Health, Bethesda, MD

Cyclin dependent kinase 5 (Cdk5) and its activators, p35 or p39, are expressed in the lens and corneal epithelial cells where they play a role in regulating cell adhesion and migration. To better understand Cdk5 functions in these epithelial cells, we screened a yeast two hybrid library from embryonic rat lens using Cdk5, p35, and p39 as baits. This screen identified myosin essential light chain (MLC) as a potential binding partner of p39. This interaction was confirmed by isolating a protein complex containing GST-MLC and p39 (GST-pulldown). Pull-down experiments with p39 deletion constructs mapped the binding site for MLC to the p39 N-terminus. Co-transfection of fluorescence-tagged constructs of MLC and p39 showed colocalization of these proteins at cell boundaries and along cytoplasmic filaments, presumably cytoskeleton. Immunoprecipitation and immunoblotting of rat lens extracts confirmed that endogenous MLC and p39 form a protein complex. Immunoprecipitating lens extracts with antibody to non-muscle myosin heavy chain (MHC II-B) showed that myosin interacts with both p39 and muskelin, a protein that binds the p39 C-terminus. Although we did not detect endogenous Cdk5 in immunoprecipitates of MHC or MLC from lens, HA-Cdk5 and GFP-MLC co-immunoprecipitated from cotransfected cells, suggesting that Cdk5 may only transiently bind to the myosin-p39 complex *in vivo*. A potential substrate for Cdk5 in this complex is the alternatively spliced form of MHC II (MHC II-B1), a known substrate of Cdk5 in the brain. RT-PCR demonstrated that this isoform is also expressed in the lens. These data support the view that p39 may form a protein complex linking muskelin to myosin and thus to the cytoskeleton. Such a complex may position Cdk5 to phosphorylate MHC II-B1 or other cytoskeletal proteins involved in cell adhesion and movement.

956

### Identification and Characterization of an Unconventional Protein Kinase Localized to the Contractile Vacuole and Golgi Complex in *Dictyostelium Discoideum*

V. Betapudi, L. S. Licate, T. T. Egelhoff; Physiology and Biophysics, Case Western Reserve School of Medicine, Cleveland, OH

We have identified a novel unconventional protein kinase in *Dictyostelium discoideum* which carries the same conserved class of “alpha kinase” catalytic domain as previously reported in myosin heavy chain kinases (MHCKs) in this amoeba, but which has a completely novel domain organization. This protein of 625 amino acids contains an N-terminal von Willebrand Factor A (vWFA) motif (also known as beta-integrin motif), and is therefore named as VwkA. VwkA$^{-}$ and overexpressing (VwkA$^{++}$) cells showed reduced growth, multinucleation in suspension culture and failure to development and gene disruption lines (*vwkA*- cells) display a developmental delay. VwkA is cytosolic in distribution, but enriched on the membranes of the contractile vacuole and Golgi-like structures in the cell. Both VwkA++ and *vwkA*- cells display altered osmotic shock responses. Despite sequence similarity to MHCKs, the purified protein failed to phosphorylate myosin II in vitro, but overexpression and gene disruption showed a significant effect on myosin II assembly levels in vivo, suggesting a possibly indirect or upstream role for VwkA in modulating myosin II localization behavior.

## Tubulin (957-965)

957

### Cryo EM Studies of Effects on Microtubule Structure by Vinblastine-Tau

R. A. Santarella,[1] J. J. Correia,[2] S. Lobert,[2,3] A. Hoenger[1]; [1]EMBL, Heidelberg, Germany, [2]Biochemistry, Univ. Miss. Medical Center, Jackson, MS, [3]School of Nursing, Univ. Miss. Medical Center, Jackson, MS

Vinblastine is a destabilizer of microtubules that favors formation of spiral protofilament polymers. It is hypothesized that at $IC_{50}$ concentrations only microtubule ends are targets of drug binding leading to curved or open structures. To investigate this we have performed cryo-electron microscopy (cryo EM) and 3-D image analysis of vinblastine binding to microtubules +/- taxol in the presence and absence of tau (Htau40). Vinblastine alone extensively destabilizes MTs and promotes small curved oligomer formation. In the presence of taxol, vinblastine promotes formation of MTs with 15 or more protofilaments and reconstruction reveals altered subunit packing. MT ends exhibit prominent spaying of short protofilaments. In the presence of vinblastine and tau, long curved spirals and bundles of spirals grow prominently from taxol-MT ends. The background is also full of short curved oligomers, straight twisted protofilament rods, and large circular structures. The alterations at MT ends are observed even at low vinblastine concentrations when tau is present, as verified by metal shadowing experiments. This indicates that tau (and MT stabilizing proteins like tau) enhances growth of vinblastine spiral polymers at MT ends. We suggest that this is the mechanism by which vinblastine selectively disrupts MT growth and dynamics.

958

### A Novel Role for the βIII Isotype of Tubulin

P. Joe,[1] C. Walss-Bass,[2] O. Pressley,[3] I. Yeh,[3] R. Luduena[1]; [1]Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX, [2]Psychiatry, University of Texas Health Science Center at San Antonio, San Antonio, TX, [3]Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX

Vertebrate tubulin is an α/β heterodimer; both α and β consist of numerous isotypes that are highly conserved in evolution. The βIII isotype has an unusual distribution of cysteine residues. It lacks the highly conserved cys239, whose sulfhydryl group can be easily oxidized to inhibit microtubule assembly. On the other hand, βIII contains the unusual cys124, located close to the conserved cys127 and cys129. βIII is known to be abundant in neurons and to occur in colon epithelia. Using a βIII-specific monoclonal antibody, we have localized βIII to a variety of cells and tissues, including islets of Langerhans, ovarian follicles, Sertoli cells, megakaryocytes, and others, all of which contain high levels of free radicals (FR) and/or reactive oxygen species (ROS). We have found that treatment of neuroblastoma cells with glutamate, which induces NO production, causes a 67 % increase in βIII levels in 15 minutes. Treatment of MCF-7 breast cancer cells with diamide, a reagent that induces disulfide bridge formation in proteins, causes a five-fold increase in the level of βIII. Our results are consistent with the hypothesis that βIII may protect microtubules from the effects of FR and ROS. Since mitochondria are major producers of ROS, our results are also consistent with the finding of

Carré et al (*J. Biol. Chem.* **277**, 33644-33669 (2002)) that βIII is present in the mitochondrial membranes of neuroblastoma cells. (Supported by grants to RFL from the Welch Foundation (AQ-0726), and the US Army (W81XWH-04-1-0231, W81XWH-05-1-0238), to CWB from the Stanley Medical Research Institute, and to ITY from the San Antonio Cancer Institute (P30 CA54174)).

959

**Multipolar Mitotic Spindles May be Important for the Antimitotic Actions of Chemically Diverse Microtubule Stabilizers**

E. A. Clark, S. L. Mooberry; Department of Physiology and Medicine, Southwest Foundation for Biomedical Research, San Antonio, TX

Chemically diverse microtubule targeting agents interrupt mitosis and lead to initiation of apoptosis. A large body of evidence suggests that at the lowest effective concentrations, these drugs inhibit normal microtubule dynamics leading to mitotic arrest. Many investigators have noted that mitotic spindles formed in the presence of these drugs can be abnormal, specifically forming multipolar spindles. We evaluated the role of these multipolar spindles in the antiproliferative effects of paclitaxel, epothilone B, laulimalide, and taccalonolide A. Dose response curves were generated for each drug in three cell lines and the $IC_{25}$, $IC_{50}$, $IC_{75}$ and 5 x $IC_{50}$s determined. The lowest concentration causing total $G_2$/M arrest was identified using flow cytometry. It was significantly higher than 5 x $IC_{50}$. Cells were treated with the entire range of concentrations, and microtubules visualized. The percentage of cells in mitosis with bipolar or multipolar spindles was determined. The results showed a concentration dependent increase in the percentage of mitotic cells with multipolar spindles. These multipolar spindles occurred at low antiproliferative concentrations. At the $IC_{75}$, 50% or more of the mitotic cells had multipolar spindles and at 5 x $IC_{50}$, essentially 100% of the spindles were multipolar. To evaluate the timing of multipolar spindle formation, HeLa cells were synchronized by double thymidine blockade, released, and treated with paclitaxel at the lowest concentration that causes $G_2$/M arrest. Multipolar mitotic spindles were observed within 4 hr, at the transition between S and $G_2$/M, suggesting that multipolar spindle formation is an early causative event. Our data show a strong link between the formation of multipolar spindles, mitotic arrest, and the antiproliferative actions of diverse microtubule stabilizers. Studies are underway to test whether the multipolar spindles are formed by normal or aberrant nucleation pathways.

960

**Activities of Dolastatin 15 Peptide Analogs as Inhibitors of Tubulin Assembly and Vinblastine Binding**

M. C. Edler, R. Bai, E. Hamel; Toxicology and Pharmacology Branch, National Cancer Institute at Frederick, Frederick, MD

Dolastatin 15 and its analogs cemadotin and tasidotin are hydrolyzed intracellularly to a common active metabolite (*N*,*N*-dimethyl-Val-Val-*N*-methyl-Val-Pro-Pro), which is significantly more potent than its precursors as an inhibitor of tubulin assembly. Assuming the metabolite binds at the "peptide site" on tubulin, it represents the least modified of the peptide and depsipeptide natural products that inhibit tubulin assembly and mitosis. Since it has long been thought that drug binding sites on tubulin may represent sites for endogenous ligands, we examined $(Val)_3(Pro)_2$ for antitubulin activity and found it to be weakly active (about 20-fold less active than the trimethylated pentapeptide). This finding led us to undertake an initial structure-function study to determine the relative importance of the three methyl groups in the dolastatin 15 metabolite, to determine whether any of the residues in $(Val)_3(Pro)_2$ could be replaced and improve activity, and whether longer peptides than $(Val)_3(Pro)_2$ would have enhanced activity. We also searched the protein data base for the $(Val)_3(Pro)_2$ motif for clues for proteins that might interact with tubulin.

961

**Sanguinarine Inhibits Cell Proliferation by Inhibiting Microtubules Assembly through Tubulin Binding**

M. Lopus, D. Panda; School of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India

We investigated the antiproliferative mechanism of action of sanguinarine, a benzophenanthridine alkaloid. Sanguinarine inhibited HeLa cell proliferation ($IC_{50}$ = 1.6 μM) by depolymerizing cellular microtubules. In the lower effective concentration range (1-3 μM), sanguinarine depolymerized microtubules of both mitotic and interphase cells. At relatively higher concentrations (> 2 μM) of sanguinarine, bundling of interphase microtubules and formation of granular aggregates were observed. The effects of sanguinarine on cellular microtubules were found to be irreversible. Sanguinarine inhibited assembly of microtubule proteins *in vitro*. It also inhibited assembly of purified tubulin. In addition, sanguinarine induced aggregation of tubulin. Sanguinarine was found to bind to tubulin and to induce changes in the secondary structure of tubulin. However, unlike several other tubulin targeting agents, sanguinarine did not arrest cells at mitosis. Further, we found that sanguinarine did not bind to the vinblastine site on tubulin. Sanguinarine exerted synergistic cytotoxic effects in combination with vinblastine. In addition, sanguinarine was found to exert synergistic cytotoxic effects on HeLa cells when used in combination with paclitaxel. The results together suggest that sanguinarine inhibits HeLa cell proliferation by perturbing microtubule assembly dynamics and that sanguinarine may have clinical potential in cancer chemotherapy. The work is supported by Department of Biotechnology, Government of India.

962

**Synthetic Peptides as Probes to Disrupt or Mimic the Interface between Tubulin and Gsα**

W. Saengsawang, B. T. Layden, T. Kobayashi, M. M. Rasenick; Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL

Gsα has been shown to interact with tubulin with high affinity ($K_D$~130nM) and increase microtubule dynamics *in vitro* by stimulating tubulin GTPase. Conversely, tubulin appears to transfer its GTP to Gsα (transactivation) resulting in GPCR-independent activation of adenylyl cyclase. The potential domains on Gsα that interact with tubulin have been identified and the interface between these proteins has been modeled. The objective of this study is to develop peptides corresponding to Gsα sequences articulating between Gsα and tubulin. A peptide array membrane corresponding to tubulin interaction domains on Gsα as well as corresponding domains on Gtα (which does not bind to tubulin) were created to investigate the specific binding site of Gsα to tubulin. Peptides from these regions were synthesized. Surface Plasmon Resonance was used to determine the binding affinity of these peptides to tubulin. Photoaffinity labeling with the GTP photoaffinity analog P3-1,4-azidoanilido-P1-5'-GTP (AAGTP) was used to study the effect of these peptides on nucleotide bound tubulin and on the transactivation of Gsα. We found 14 peptides from Gsα that show binding to tubulin compared to the corresponding Gtα peptide. We synthesized peptides from the switch II and switchIII regions of Gsα. We found that these peptides bind to tubulin with $K_D$~5μM. Interestingly, each of these peptides cause tubulin to lose its nucleotide in concentration dependent manner and block transactivation of Gsα. These results suggest that Gsα derived peptides bind to tubulin and alter the affinity of nucleotide binding to tubulin. This study will provide novel insight into domains on Gsα that interact with and/or alter the nucleotide

binding site on tubulin. It will also aid in an understanding the interface between G protein signaling and the cytoskeleton. Support NIMH MH39595

963

### Tissue Distribution of the βV Isotype of Tubulin

I. Yeh,[1] A. Banerjee,[2] O. Pressley,[1] J. De Luna,[2] R. F. Luduena[2]; [1]Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX, [2]Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX

Of the various isotypes of β-tubulin, perhaps least is known about βV. In order to examine this further, we have developed a monoclonal antibody specific for βV. We have surveyed various tissues with this antibody and found that βV is present in a relatively small number of cell types. These include the islets of Langerhans, intestinal glands, macrophages, megakaryocytes, and glia. In cultured cells, βV is strongly present in C6 glioma and PC3 prostate cancer cells, but only weakly in MCF-7 breast cancer and HeLa cells. However, in formalin fixed, paraffin embedded sections, we have found strong βV staining in a low-grade breast ductal carcinoma *in situ* (DCIS), but not in a high grade DCIS. Interestingly, βIII was present in both of these. The tissue distribution of βV is strikingly similar to that of βIII. These tissues are rich in free (FR) radicals and reactive oxygen species (ROS). Notably, both βV and βIII contain a cysteine cluster at positions 124, 127 and 129 and lack the highly oxidizable cys239 found in most of the other β isotypes. (Supported by grants to RFL from the Welch Foundation (AQ-0726), and the US Army (W81XWH-04-1-0231; W81XWH-05-1-0238) and to ITY from the San Antonio Cancer Institute (P30 CA54174)).

964

### Purification and Characterization of Tubulin from *Agaricus bisporus*

B. Arthur, K. M. Middleton, A. S. Davis; Cytoskeleton Inc, Denver, CO

The incidence of invasive fungal infections in humans has risen dramatically over the past twenty years. In addition to human pathologies, numerous fungal pests threaten agricultural crops. Anti-fungal drug development for medical and agricultural use has been limited by the inability of these compounds to target pathogens specifically, subjecting the host to few negative effects. One drug target is tubulin, a cytoskeletal protein that is essential for both cell division and survival. Targeting of this protein for drug development is complicated by the fact that tubulin orthologs are expressed in all eukaryotes. It follows that for an anti-fungal tubulin drug to be efficacious, it must show high specificity for the pathogenic tubulin. A principal obstacle in determining the species specificity of anti-tubulin compounds is the lack of an *in vitro* system wherein the drug's effect on tubulin from different species can be compared. Here we describe the purification and characterization of tubulin from the fungus *Agaricus bisporus.* Tubulin was purified and polymerization conditions were established resulting in microtubules that ranged from 8 to 15 µm. The critical concentration was 0.3 mg/ml, lower than that of bovine tubulin. These conditions yielded 0.9 mg/ml of polymer/$OD_{340}$/cm, compared to 5.0 mg/ml of polymer/$OD_{340}$/cm for bovine tubulin. Compounds that act as polymerization inhibitors, such as rhizoxin (10 µM), or stabilizers, such as paclitaxel (10 µM), were found to decrease and enhance polymerization, respectively. This system can be used to determine the activity of a candidate compound on fungal tubulin polymerization. In future studies, drug activity determinations for *A. bisporus* tubulin will be conducted in parallel with mammalian tubulin, thereby allowing comparisons to be made regarding the compound's specificity for tubulin from different species.

965

### Characterization of NUD-1 Interactors in *C elegans*

J. G. Phillips, R. Thomas, M. Till, K. Peters, G. A. Caldwell, K. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL

NUD-1 is the *C. elegans* ortholog of a conserved family of proteins originally identified in a screen for proteins affecting nuclear positioning within the hyphae of the filamentous fungus *Aspergillus nidulans*. We have previously shown functional conservation between *A. nidulans* NUDC and NUD-1, as well as evidence that the worm *nud-1* gene is expressed in a variety of *C. elegans* cell types, including neurons, mutlinucleated tissues, and mitotically dividing cells. The *C. elegans* one-celled embryo represents an excellent system by which cellular components involved in the cytoskeleton can be elucidated. In this regard, we report evidence that gamma-tubulin is not localized to centrosomes in *nud-1* (RNAi) *C. elegans* embryos. These data are corroborated by mammalian studies wherein NudC has been shown to interact with dynein and to colocalize with p150dynactin and with gamma-tubulin. It is thought that NUD-1 probably interacts with a variety of proteins; the structure of NUD-1, which contains putative protein interaction domains, is suggestive of this functionality. Current work is directed toward investigating the relationship between gamma-tubulin, NUD-1, and other potential interacting proteins using a combination of biochemical analysis and immunocytochemistry.

## Dynein II (966-988)

966

### BOP2 Encodes a Novel WD-Repeat Protein that Regulates Inner Arm Dynein Activity

C. A. Perrone,[1] D. Tritschler,[1] R. Bower,[1] E. T. O'Toole,[2] M. J. Wargo,[3] E. F. Smith,[3] M. E. Porter[1]; [1]Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN, [2]Molecular, Cellular, & Developmental Biology, University of Colorado, Boulder, CO, [3]Biological Sciences, Dartmouth College, Hanover, NH

To identify novel components involved in the regulation of flagellar motility, we screened a collection of motility mutants generated by insertional mutagenesis. Analysis by thin section electron microscopy and image averaging identified three slow swimming strains that share similar defects in the assembly of a distally located inner arm structure. Recovery of genomic DNA flanking the site of plasmid insertion and RFLP mapping demonstrated that all three strains are mutant alleles at the *BOP2* locus. Rescue by transformation revealed that *BOP2* expresses an ~5kb transcript encoding a highly conserved, 145 kD, WD-repeat containing polypeptide. Western blot analysis of isolated axonemes and dynein extracts showed that BOP2 co-fractionates with a subset of inner arm subunits. Sequencing of the original *bop2-1* allele (isolated as a suppressor of *pf10*, Dutcher et al., 1988) identified a single base pair change in a splice acceptor site. The mutation results in a complex pattern of alternative splicing at cryptic splice sites downstream, and the new transcripts contain premature stop codons. These results suggest that *bop2-1* is effectively a null allele, consistent with the absence of a BOP2 signal on Western blots. Analysis of microtubule sliding patterns and velocities further demonstrate that the

regulation of dynein activity on specific doublets is defective in *pf10* axonemes. However, microtubule sliding shifts to a more wild-type-like pattern in *bop2 pf10* double mutants. Collectively, these results suggest BOP2 is an inner arm associated polypeptide involved in regulating microtubule sliding on specific subsets of doublet microtubules (Supported by the NIH).

967

**Localization of a Regulatory Intermediate Chain (IC) Complex at the Base of the I1 Dynein**

R. Bower,[1] C. A. Perrone,[1] J. Mueller,[1] E. T. O'Toole,[2] M. Wirschell,[3] L. Fox,[3] W. S. Sale,[3] M. E. Porter[1]; [1]Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN, [2]Molecular, Cellular, & Developmental Biology, University of Colorado, Boulder, CO, [3]Cell Biology, Emory University School of Medicine, Atlanta, GA

Our goal is to understand how the *Chlamydomonas BOP5* gene product, the IC138 phosphoprotein, contributes to the assembly and regulation of the I1 inner arm dynein (Hendrickson et al., 2004). Here we focus on an unusual *bop5* motility mutant, 6F5, isolated by insertional mutagenesis. Southern blots and PCR showed that >90% of the *IC138* gene is deleted in 6F5. In backcrosses to a *nit1* strain with wild-type motility, the motility defect co-segregates with the absence of IC138. To assess how the loss of IC138 affects the other I1 subunits, we analyzed 6F5 axonemes on Western blots. Most null mutations in other dynein ICs block complex assembly. Contrary to expectation, most I1 subunits are present in 6F5 axonemes, but both IC138 and IC97 are missing (see also Wirschell et al., these proceedings**)**. Fractionation of 6F5 dynein extracts by sucrose density gradient centrifugation revealed that the remaining I1 subunits are dissociated into two distinct peaks. IC138 and IC97 are therefore components of the I1 dynein, but not required for its assembly in the axoneme. Analysis of 6F5 axonemes by image averaging of longitudinal sections revealed a defect at the base of the I1 dynein, in close proximity to radial spoke 1 and outer dynein arms. Microtubule sliding velocities are also reduced in 6F5 axonemes. Transformation with a wild-type copy of *IC138* restores assembly of both IC138 and IC97 and increases sliding velocities to wild-type levels. Taken together, these observations are consistent with the hypothesis that IC138 (and possibly IC97) play a central role in mediating signals between the radial spokes and dynein arms (supported by NIH).

968

**25 Dynein Heavy Chains in *Tetrahymena thermophila*: Evidence that the Dynein Family Evolved in Two Stages**

D. E. Wilkes, H. Watson, D. J. Asai; Department of Biology, Harvey Mudd College, Claremont, CA

We have identified 25 dynein heavy chain (DYH) genes that are expressed in *Tetrahymena thermophila.* These include the 14 DYH genes previously described (Asai and Wilkes, 2004, J. Eukaryot. Microbiol. 51: 23-29), and 11 new DYH genes inferred to encode single-headed axonemal dyneins. The expression of the 11 newly identified DYH genes was upregulated in cells that had been deciliated. Phylogenetic analyses of the motor domains of dyneins from *Tetrahymena* and other model organisms revealed two patterns of groupings. The DYH1-7 sequences group with orthologous sequences from other organisms. In contrast, DYH8-25 mostly grouped in organism-dependent clusters. For example, there is a large clade of protistan (*Chlamydomonas* and *Tetrahymena*) dyneins, there are clades of only *Tetrahymena* dyneins, and there are clades of only animal dyneins. These results suggest that the present family of dynein heavy chains evolved in two stages. DYH1-7 plus the ancestor of the DYH8-25 genes emerged early, prior to the divergence of the last common ancestor of extant eukaryotes (see D.R. Mitchell, 2004, Biol Cell 96: 691-696). In *Tetrahymena*, these functionally specialized "foundation" dyneins comprise two non-axonemal dyneins (*Tetrahymena* DYH1 and DYH2), three-headed ciliary outer arm dynein (DYH3-5), two-headed inner arm dynein (DYH6,7), and a single-headed inner arm dynein. The second stage involved the duplication and subsequent divergence of the single-headed dynein, resulting in DYH8-25; the second stage occurred after the radiation of organisms. Perhaps the additional dyneins enabled the organism to further specialize axonemal motility and/or rapidly form new cilia. Supported by grants from the National Science Foundation and the Howard Hughes Medical Institute.

969

**Identification of a Third Member of the LC8 Family of Dynein Light Chains in *Chlamydomonas* Flagella**

P. Rompolas,[1] R. S. Patel-King,[1] O. Gorbatyuk,[1] K. Wakabayashi,[1] G. J. Pazour,[2] S. M. King[1]; [1]Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, CT, [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

Members of the LC8 family of dynein light chains (LCs) are highly conserved from algae to humans, and are thought to play an essential role in assembly of the dynein motor complex. LC8 and LC6, the founding members of this family, were initially identified in *Chlamydomonas* outer dynein arms. Analysis of the *Chlamydomonas* genome identified a gene that encodes a third member of this family (here termed LC10) which shares 48 and 28% identity with *Chlamydomonas* LC8 and LC6, respectively. LC10 contains 103 amino acids with a molecular mass of 12,086 Da and a pI of 5.28. Northern blotting identified a single 1.2 kb mRNA that is highly up-regulated upon deflagellation. Immunoblot analysis demonstrated that an antibody raised against a MBP/LC10 fusion protein reacted strongly with LC10, but specifically did not detect either LC6 or LC8. Furthermore, we found that LC10 is a flagellar component that is not solubilized by detergent treatment, but is readily extracted from axonemes by 0.6 M NaCl. In addition, flagella from the mutants *oda1* (missing outer arms and the docking complex) and *oda2* (missing only outer arms) were found to completely lack LC10. This strongly suggests that LC10 is an integral component of the outer dynein arm. The LC10 gene is located close to the *ODA12* locus that encodes the essential outer arm protein LC2; this gene is missing in the *oda12-1* insertional allele, but not in *oda12-2*. Transformation of *oda12-1* with the LC2 gene alone restores outer arm assembly (Pazour et al [1999] Mol Biol Cell 10, 3507-3520). We are currently using this LC10-deficient strain to assess whether the lack of LC10 has consequences for outer arm dynein function.

970

**Association of the Cytoplasmic Dynein Intermediate Chain Isoforms**

K. W. Lo, K. Pfister; Cell Biology, University of Virginia, Charlottesville, VA

The cargo binding domain of Cytoplasmic Dynein 1, the multi-subunit protein complex responsible for many different microtubule-based intracellular movements, consists of dimers of five subunits; intermediate chains, light intermediate chains, and three families of light chains. Alternative splicing of the two intermediate chain genes results in six protein isoforms. It has been hypothesized that different intermediate chain isoforms may regulate specific dynein functions. However, only minimal data on the potential combinations of the intermediate chain isoforms in

the dynein complex has been available. To determine if different intermediate chains are able to associate, pairs of epitope-taged intermediate chains were expressed in cultured cells. Co-immunoprecipation analyses of the transfected proteins demonstrate that the six intermediate chains form all combinations of homodimers and heterodimers. Therefore the formation of dynein complexes with different combinations of isoforms is not limited by interactions between the various intermediate chains. We next sought to map the domain necessary for the dimerization of the intermediate chains. A series of epitope-tagged truncation mutants of intermediate chain 2C, were expressed in cultured cells. Co-immunoprecipitation analyses showed that the region necessary for dimerization is restricted to amino acids 150 - 250 of the intermediate chain. This amino acid sequence does not include either of the two intermediate chain regions with motifs that are often involved in protein-protein interactions, the N-terminal coiled-coil and the C-terminal WD repeat domains. This sequence is also downstream from the binding domains for the LC8 and Tctex1 light chains. Therefore binding of those two light chains is not required for intermediate chain dimerization. Interestingly, this amino acid sequence does include the Roadblock light chain binding domain, and we are investigating the importance of the Roadblock light chains for the dimerization of the intermediate chains in the dynein complex.

971

**Complexity of Cytoplasmic Dynein Light Chain Expression in COS-7 Cells**

S. L. Bird, P. S. Vaughan, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

Cytoplasmic dynein is an abundant microtubule motor protein in COS-7 cells and is responsible for the transport of multiple cargos during interphase and mitosis. Similar to other cytoplasmic dynein subunits, multiple isoforms of the Tctex-1, LC8 and LC7 light chains (LCs) have been identified and characterized. We are interested in how many populations of dynein are needed to accomplish all dynein functions in COS-7 cells and analysis of the LCs is complicated by the possibility of LC isoform expression. Because COS-7 cells have been shown to express a limited subset of IC isoforms (Vaughan et al., 2001), we explored the complexity of LC expression with the goal of performing immunofluorescence microscopy and siRNA analysis on each LC class. We utilized RT-PCR based on known sequence information to amplify light chain cDNA sequences from COS-7 cell mRNA. We then employed cloning, sequencing, and sequence comparisons to identify which LC isoforms were expressed. Because variable expression of each isoform was possible, we analyzed 9 independent isolates for LC8, 10 for Tctex-1 and 9 for LC7. Similar to our findings for other cytoplasmic dynein subunits, COS-7 cells express a single isoform of the Tctex-1, LC8 and LC7 LCs. This suggests that a single population of cytoplasmic dynein is capable of executing all interphase and mitotic dynein functions. For siRNA analysis, we have identified promising siRNA targets using sequence scanning programs and designed and created siRNA-expressing plasmid constructs. This analysis resulted in the creation of four different target siRNA sequences for each LC, which allows comprehensive analysis of LC depletion. In conclusion, COS-7 cells express a limited number of LC isoforms which simplifies our analysis of LC function and targets for siRNA analysis. (Supported by NSF-REU Program and NIH GM60560).

972

**Complexity of P150$^{Glued}$ Phosphorylation**

L. Casaletti, P. S. Vaughan, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

A growing number of microtubule (MT) binding proteins display a behavior known as "tip-tracking" when they are expressed as GFP fusion proteins (Vaughan, 2004). Recent work on the p150$^{Glued}$ subunit of dynactin suggests that tip-tracking reflects a dynamic association with MTs which is released by phosphorylation. Although several lines of evidence suggest that members of the PKA family are involved, other kinases have also been implicated. The mammalian target of rapamycin (mTOR) was identified recently as another potential kinase that regulates MT tip-tracking proteins (Choi et al., 2002). We have assessed the role of mTOR phosphorylation on p150$^{Glued}$, EB1 and CLIP-170 by: 1) 2-D gel analysis of control cells and cell treated with rapamycin, 2) immunofluorescence and live-cell imaging of cells expressing GFP-p150$^{Glued}$ or EB1-GFP after treatment with rapamycin, and 3) imaging of cells over-expressing wild-type mTOR, mTOR kinase-dead mutants and rapamycin-resistent mTOR mutants. These findings reveal that mTOR has a differential effect on p150$^{Glued}$ and EB1. Based on the results of rapamycin treatment, it appears that mTOR activity promotes the binding of p150$^{Glued}$ to microtubules (in contrast to PKA) but directs the release of EB1 from MTs. This effect on p150$^{Glued}$ appears to be indirect, because inhibition of mTOR with rapamycin results in an increase in p150$^{Glued}$ phosphorylation complexity by 2-D gel analysis. To dissect these findings, we performed *in-vitro* microtubule-binding assays using recombinant p150$^{Glued}$ (1-330) in the presence of control or rapamycin-treated COS-7 cell extracts. Rapamycin-treated extracts decreased the affinity of p150$^{Glued}$ for MTs (via p150$^{Glued}$ phosphorylation) and this effect was not influenced by site-directed mutations at other phosphorylation sites. This work suggests that multiple pathways regulate p150$^{Glued}$. (Supported by NIH and DOD grants to KTV).

973

**Cytoplasmic Dynein Phosphorylation Changes Subunit Composition and Regulates Dynein Localization During Mitosis**

J. Whyte,[1] W. S. Lane,[2] K. Pfister,[3] S. M. King,[4] P. S. Vaughan,[1] K. T. Vaughan[1]; [1]Biological Sciences, University of Notre Dame, Notre Dame, IN, [2]Biological Sciences, Harvard University, Cambridge, MA, [3]Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA, [4]Biochemistry, University of Connecticut Health Center, Farmington, CT

Previous work identified a phosphorylation site in the dynein intermediate chains (ICs) which regulates binding to the putative receptor complex dynactin (Vaughan et al., 2001). To identify dynein kinases, we examined mitotic cytoplasmic dynein and determined that the ICs are phosphorylated by gel-shift and 2D gel analysis. ICs were subjected to MS/MS analysis revealing two novel phosphorylation sites. T89 falls within the binding domain for dynactin, and site directed mutants designed to mimic a phosphorylated state reduce dynactin binding significantly. T89 fits the consensus for p38MAPK and phosphorylation by p38MAPK is specific for this site *in vitro*. Inhibition of p38MAPK results in premature redistribution of dynein from kinetochores to spindle poles and results in lagging chromosomes during anaphase. Y130 falls within a domain implicated in LC8 (a dynein light chain) binding and fits the consensus for phosphorylation by *c-abl* kinase. *C-abl* phosphorylates wild-type but not Y130F mutant ICs *in vitro*. This phosphorylation was blocked by treatment with Gleevec (a *c-abl* inhibitor) and Gleevec treatment of live cells also blocked phosphorylation at Y130, suggesting that *c-abl* is a mitotic dynein kinase. Binding assays to test the impact of Y130 phosphorylation demonstrated that phosphorylated ICs bind less LC8 than dephosphorylated ICs. Immunostaining with antibodies generated against the phosphorylated ICs reveal that this phospho-isoform is not present in interphase cells. Instead these antibodies label dynein at the cell cortex in

mitosis and this labeling becomes prominent during late stages of mitosis. Interestingly, phospho-Y130 antibodies also brightly label the congressing furrows of cells undergoing cytokinesis. These results suggest that phosphorylation of ICs modulates subunit composition during mitosis and plays a role targeting dynein to multiple functional sites during mitosis. Supported by NIH GM60560.

974

**Alternative Splicing as a Mechanism to Modulate the Specificity of Cytoplasmic Dynein Phosphorylation**

S. Tauhata,[1] J. Burke,[1] E. Joseph,[1] W. S. Lane,[2] S. M. King,[3] K. Pfister,[4] P. S. Vaughan,[1] K. T. Vaughan[1]; [1]Biological Sciences, University of Notre Dame, Notre Dame, IN, [2]Biological Sciences, Harvard University, Cambridge, MA, [3]Biochemistry, University of Connecticut Health Center, Farmington, CT, [4]Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA

Although common in higher eukaryotes, the function of alternative splicing is not understood completely. Analysis of the cytoplasmic dynein intermediate chains (ICs) in neurons reveals expression of six isoforms derived from alternative splicing of two gene products. Because this splicing is restricted to the binding domain for the p150$^{Glued}$ subunit of dynactin, we developed binding assays using each IC isoform and determined that these isoforms share similar affinity and specificity for p150$^{Glued}$. To explore differences between isoforms, we performed MS/MS analysis on each polypeptide to map phosphorylated residues. Each isoform displayed phosphorylation in the p150$^{Glued}$-binding domain of the ICs, however the specific sites of phosphorylation were unique to each isoform. In addition, the sites fit consensus sequences for different kinases suggesting that each dynein subpopulation is regulated by an independent signaling pathway. Because the binding sites for two dynein light chains (LCs) are in close proximity to the phosphorylation sites, we compared the binding affinities of ICs alone and ICs with either a full or partial LC complement. The presence of LCs in the IC preparations did not improve the affinity of ICs for p150$^{Glued}$ nor did it change the binding specificity. In mixtures of IC isoforms however, the presence of LCs drove formation of IC homodimers rather than heterodimers suggesting a role in isoform sorting and selection during complex assembly. These studies suggest that alternative splicing of the dynein ICs presents different recognition sequences for dynein kinases and directs phosphorylation by isoform-specific kinases. The presence of dynein LCs drives the formation of isoform homodimers which ensures that each dynein molecule is regulated by a single kinase. Given the similarities to other neuronal microtubule motors, this could be a universal mechanism to enhance regulatory diversity in neurons.

975

**A Novel Microtubule-Binding Domain within Dynactin Increases the Processivity of Cytoplasmic Dynein**

S. J. King, T. L. Culver, S. A. Lex; School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO

The multiple roles that the cytoplasmic dynein activator dynactin plays inside cells require dynactin molecules to bind different regions of cellular microtubules and with different physicochemical properties to those microtubules. Here, we discovered that dynactin contains a novel microtubule-binding domain in addition to a previously described CAP-Gly domain. The newly identified domain is highly basic and is present only in the dynactin members of the CAP-Gly family of proteins. Using single-particle assays, we found that the dynactin CAP-Gly domain remains firmly attached to a single point on a microtubule. Interestingly, the dynactin basic domain progressively moves along the microtubule in the absence of any molecular motor, a process we have termed skating. To determine the physiological relevance of microtubule skating we performed motility assays with beads coated with both the motor cytoplasmic dynein and p150 polypeptides that contained one of the two microtubule-binding domains. We found that the p150 basic domain increased the processivity (run length) of cytoplasmic dynein motility events. In contrast, the p150 CAP-Gly domain did not enhance cytoplasmic dynein motor processivity. Our data suggest that the ability of individual dynactin molecules to skate along microtubules is utilized by cytoplasmic dynein as a tether to maintain contact with the microtubule cytoskeleton, thereby increasing dynein processivity. The dynactin skating behavior is the first description of a motor-independent mobile binding interaction between a protein and a biological polymer outside of DNA/sliding clamp interactions and may thus herald a new mechanism for dynamic protein/protein interactions.

976

**Impact of Transfected Cytoplasmic Dynein Subunits on Dynein-based Transport**

W. L. Towns, P. S. Vaughan, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

Cytoplasmic dynein is a microtubule motor protein responsible for the transport of multiple cargos. In previous work, we mapped a phosphorylation site (Ser-84) in the dynein intermediate chains (ICs) and determined that this phosphorylation modulated the binding of the ICs to dynactin. To test if IC phosphorylation affected additional aspects of dynein function, we prepared GFP-tagged versions of wild-type and truncated ICs, as well as mutants mimicking the dephospho- and phospho-states. Using cell lysates from IC-transfected cells, we utilized immunoprecipitation analysis and sucrose gradient analysis to show that IC phosphorylation does not appear to affect other dynein activities such as dynein complex assembly and dynein light chain (LC) recruitment. We have compared our results using alternative methods of cell lysis to test if IC transfection has an impact on dynein complex stability. Sucrose density gradient sedimentation analysis of "osmotically swollen" lysates produces IC peaks that are markedly sharper and more shifted towards 20S fractions compared to detergent lysis. Immunoprecipitates from these 20S fractions with anti-GFP reveals that transfected IC-GFP incorporates into dynein and co-assemble with native ICs less effectively than 20S fractions from detergent lysates regardless of phosphorylation state, or the presence of the WD40 repeat domain. We also probed the osmotic lysate 20S immunoprecipitates for the Tctex-1, LC8, and LC7 LCs. Wild-type as well as phospho-mimicking ICs displayed little if any binding of each LC class. These results suggest that transfected IC constructs mediate their dominant negative effects primarily through saturation of dynein receptors rather than through disruption on native dynein complexes. (Supported by the AHA and NIH).

977

**Differential Light Chain Assembly Influences Outer Arm Dynein Motor Function**

L. M. DiBella,[1] O. Gorbatyuk,[1] M. Sakato,[1] K. Wakabayashi,[1] R. S. Patel-King,[1] G. J. Pazour,[2] G. B. Witman,[3] S. M. King[1]; [1]Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, CT, [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, [3]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA

The intermediate chain/light chain (IC/LC) complex of *Chlamydomonas* outer arm dynein contains at least six distinct LC species. The *oda6*

mutant is unable to assemble outer arms due to the lack of IC2. An intragenic pseudorevertant at the *oda6* locus (*oda6-r88*) contains 23 residues altered from wildtype and can assemble outer arms (Mitchell & Kang [1993] J Cell Sci 105, 1069-1078). However, this strain exhibits significantly reduced flagellar beat frequency (~30 Hz), whereas a second pseudorevertant (*oda6-r75*), containing 10 altered residues in a region of IC2 adjacent to the *oda6-r88* deficiency, appears essentially wildtype (~55 Hz). Immunoblot analysis revealed that LC2 (Tctex2), LC6 (related to the highly conserved LC8 protein) and LC9 (Tctex1) are specifically missing in *oda6-r88*, but not in *oda6-r75*. The *oda6-r88* motility phenotype is not due to the lack of LC6 alone, as the LC6 null mutant (*oda13*) exhibits only a minor swimming abnormality. Intriguingly, we found that the LC2 null mutant (*oda12-1*) assembles some outer arm proteins; however, they are apparently non-functional as beat frequency of this strain is only ~20 Hz. This suggests that there is a functional difference between dyneins assembled in the absence of LC2 (from *oda12-1*) compared to dyneins that merely do not incorporate this protein (from *oda6-r88*). Furthermore, o*da6-r88* is not an extragenic suppressor of *oda12-1*, as the double mutant has a beat frequency similar to *oda12-1*. We did find that *oda12-1* dynein contains LC6 and minor amounts of LC9, raising the possibility that assembly of LC6 in the absence of LC2 may be detrimental. These data suggest that LC2, LC6 and LC9 have different roles in outer arm assembly and that one or more of these proteins is required for wildtype motor function in the *Chlamydomonas* flagellum.

978

**Stepwise Movements of the Motor Proteins in a Breast Cancer Cell**

T. M. Watanabe; Center for Interdisciplinary Research, Tohoku University, Sendai, Japan

Motor proteins, myosin, kinesin and dynein, involve in membrane transport in cells. *In vitro* motility assay and single-molecule measurement technology have revealed the motile mechanism of the motor proteins. However, in the living cell, there are many additional factors to consider apart from the condition *in vitro*; for example, ionic strength, adaptor proteins, cytoskeleton, viscoelasticity. The direct observation of the movements of the motor protein in cell is necessary to understand the molecular mechanism of the transportation. Here, we tried to observe the elements of the movements of the motor proteins in living cell using new techniques. To observe the movement of motor proteins in a cell for long time and with high temporal resolution, stable and intense Quantum dots (QD) labeled to HER2 (Human EGF Receptor 2) via antiboby (antiHER2-QD) was encytosed into a breast cancer cell in which HER2 was overexpressed. We observed the transportation of antiHER2-QD in three dimensions (3D) using new confocal microscopic system, which could obtain one set of 9 conforcal images at serial focal positions within 330ms. As a result of 3D observations, after endocytosis, antiHER2-QD moved along the membrane, and then was transported towards the nucleus. The movement along the membrane was related to the actin-dependent motor, most likely myosin VI, and the transportation toward the nucleus was related to dynein. Next, we measured each movement at nm, ms revel using modified-FIONA (Fluorescence Imaging with One Nanometer Accuracy) method. The movement along the membrane consisted of 29 and 16 nm steps, suggesting that myosin VI in cell walks on one side of an actin filament like a ropewalker. The movement toward the nucleus consisted of successive 8 nm steps. Therefore we concluded the unitary stepsize of dynein in cell is 8 nm.

979

**Kinetic Analysis of Tail Motions of Cytoplasmic Dynein Motor Domain**

T. Mogami, T. Kon, R. Ohkura, K. Sutoh; Life Sciences, University of Tokyo, Tokyo, Japan

Cytoplasmic dynein is a microtubule-based minus-end directed motor protein and is a large multi-component protein composed of two identical heavy chains (DHC), several intermediate, light intermediate, and light chains. The motor domain is composed of the C-terminal two-thirds of the DHC (380kDa) which contains several functional domains, i.e. AAA ring, stalk and tail domains. The AAA ring contains six AAA ATPase modules arranged in a ring-like shape. The N-terminal 160kDa region extending from the AAA ring forms the tail domain, which is responsible for dimerization and cargo binding. The primary ATPase site responsible for force generation is thought to reside in the first AAA module. The question of how structural changes of DHC induced by ATP hydrolysis are coupled with force generation remains to be answered. To detect structural changes of the tail against the AAA ring of DHC, BFP-GFP-based FRET measurements have been carried out on the recombinant 380kDa motor domain. The swing-like motions of the tail induced by ATP hydrolysis were detected as FRET efficiency changes. Two FRET states, State I and State II, were detected during ATP hydrolysis. The conformational transition from State I to State II was triggered by binding of ATP, and that from State II to State I occurred possibly at the ADP-release step. The purpose of this work was to perform kinetic analysis of transitions between the two conformational states. Rates of the transitions were determined by stopped flow measurements of FRET efficiency changes. The measurements also showed that the rate of transition from State II to State I increased several-fold on addition of microtubules, whereas the rate of the other transition was not. These results were consistent with the model that the tail motion is the power-stroke of dynein motor.

980

**Single Dynein-Dynactin Complexes Exhibit Bidirectional Motion *in vitro***

J. L. Ross,[1,2] K. Wallace,[2] H. Shuman,[1,2] Y. E. Goldman,[1,2] E. Holzbaur[2,1]; [1]Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, PA, [2]Physiology Dept, University of Pennsylvania, Philadelphia, PA

Cytoplasmic dynein is a microtubule associated molecular motor essential for diverse functions in eukaryotic cells, such as retrograde axonal transport and mitosis. Dynein and dynactin complex in vivo to couple cargo to the dynein motor protein and allow transport. In order to further understand the properties of the dynein-dynactin complex, we have directly observed the motility of a GFP-labeled dynactin complexed to dynein in vitro using total internal reflection fluorescence microscopy. While motility is predominantly minus-end directed, our data shows that individual dynactin-dynein complexes display processive events (~1 um) in both minus-end and plus-end directions along microtubules under the zero-load conditions in our assay. The ability to switch direction and move bidirectionality is a unique property that may have biological relevance for transport in vivo. In addition, the velocity exhibits bi-phasic kinetics as a function of ATP that are a direct consequence of the AAA structure of the dynein head, which is distinct from other motor protein families. Supported by NIHP01AR051174

981

**Testing the Requirement of Dynein and Microtubule Depolymerization during Asymmetric Spindle Positioning of *C elegans* Embryos**

T. Nguyen-Ngoc, P. Gönczy; ISREC and EPFL, Lausanne, Switzerland

Spindle positioning is essential for spatial control of cell division, as it dictates the size and position of daughter cells. During the first mitotic division of the *Caenorhabditis elegans* embryo, the spindle elongates asymmetrically toward the cell posterior, giving rise to an unequal division along the anterior-posterior axis. Asymmetric spindle elongation is achieved by an imbalance of forces acting on the two spindle poles, with a larger net force pulling on the posterior pole (Grill et al., 2001). These pulling forces result from the action of cortical force generators, with a larger number of active cortical force generators pulling on astral microtubules of the posterior aster (Grill et al., 2003). While the generation of these pulling forces is known to require the alpha subunit of heterotrimeric G proteins and their activators (Colombo, 2003), the mechanical basis of cortical force generators is not known. By analogy with mechanisms driving chromosome segregation and after precedent in budding yeast, pulling forces could be generated by cortically tethered cytoplasmic dynein and/or microtubule depolymerization-coupled movements. To determine whether dynein is implicated, we compromised the dynein heavy chain DHC-1 using temperature sensitive mutants (Hamill et al., 2002). This allowed us to bypass the early requirements of dynein, conduct spindle severing experiments, and thus deduce the extent of pulling forces by measuring the velocities of the liberated spindle poles. Our results suggest that DHC-1 is required, at least in part, for pulling force generation. Furthermore, preliminary evidence using similar approaches indicates that microtubule depolymerization-coupled movements are implicated as well. Thus it appears that both dynein and microtubule depolymerization together generate pulling forces during asymmetric spindle positioning of *C. elegans* embryos.

982

**Microtubule Binding Allows Dynactin to Mediate Microtubule Search-Capture During Mitosis**

J. R. Bader, E. H. Hinchcliffe, P. S. Vaughan, K. T. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

Microtubule-based motor proteins are critical for many cellular processes including cell division. A number of microtubule-based motor proteins have been identified that function in mitosis, including cytoplasmic dynein. Cytoplasmic dynein and its receptor complex, dynactin, are thought to be a critical component at spindle poles, the cell cortex, and kinetochores during mitosis. Disruption of any one component is believed to lead to a missegregation of chromosomes, which is now recognized as a primary mechanism of cancer initiation. We have proposed a model of microtubule search-capture at the cell cortex and kinetochores during mitosis, which is mediated by dynactin and coupled to dynein based transport. Dynactin has two important functions during mitosis: binding to microtubules through $p150^{Glued}$ and recruitment of cytoplasmic dynein. To determine the sequence of dynactin functions, we are perturbing protein regulation, function, and expression. Previous work has shown that treatment of cells with forskolin diminishes the microtubule binding ability of the $p150^{Glued}$ subunit of dynactin during interphase. Forskolin stimulates the activity of PKA which phosphorylates the MT-binding domain of $p150^{Glued}$. Live-cell imaging of stably transfected GFP-tubulin BSC-1 cells and immunofluorescence of COS-7 cells treated with forskolin was performed to assess the role of $p150^{Glued}$ during mitosis. Loss of MT-binding through $p150^{Glued}$ resulted in multi-polar spindles, distorted spindles, and lagging chromosomes, suggesting that microtubule search-capture is inhibited at the cell cortex and kinetochores. To confirm that these observed defects are a result of diminished MT-binding through dynactin, we performed siRNA and rescue experiments which suggest multiple roles for dynactin during mitosis. Supported by NIH GM60560.

983

**Identification of Dynein/Dynactin in a Biochemical Screen for Microtubule-Actin Crosslinking Proteins and Probing its Role in Cell Motility**

O. C. Rodriguez, C. M. Waterman-Storer; Cell Biology, The Scripps Research Institute, La Jolla, CA

Interactions between microtubules (MTs) and F-actin are important for dynamic polarized cell behaviors such as directed motility. To identify candidate proteins for mediating MT/F-actin crosslinking interactions, we purified them from HeLa cells by sequential affinity to MTs and F-actin, followed by mass spectrometry. Two of the identified polypeptides were the heavy and intermediate chains of cytoplasmic dynein. Cytoplasmic dynein and its interacting partner, dynactin, are a minus-end MT motor complex that participates in many MT-based motility events. We tested the role of dynein/dynactin in mediating MT/F-actin interactions by interfering with dynein/dynactin function via p50/dynamitin overexpression in migrating Ptk1 epithelial cells and NIH3T3 fibroblasts. First we analyzed the effect on the dynamics and organization of MTs and F-actin labeled with spectrally distinct fluorophores by dual wavelength Fluorescent Speckle Microscopy. In Ptk1 cells overexpressing p50-GFP, dual-wavelength kymograph analysis of F-actin and MT movements revealed a ~30% increase in the rates of F-actin and microtubule flow in the lamella relative to control GFP-injected cells, but no obvious disruption of MT/F-actin structural interactions. Thus in Ptk1 cells dynein/dynactin inhibition did not appear to uncouple MTs from F-actin retrograde flow. We next examined the role of dynein/dynactin in centrosome orientation during cell migration. Using overexpression of another dynactin subunit, p62, we found that although p50-GFP overexpression perturbs centrosome reorientation in a wound assay of NIH3T3 cells, as was previously known, GFP-p62 overexpression did not. Current experiments focus on determining if p50 and p62 overexpression have differential effects on cell migration.

984

**The Affinity of the Dynein Microtubule-Binding Domain is Modulated by Changes in Helical Register of its Full-Length Coiled-Coil Stalk**

J. E. Garbarino, C. E. Tan, I. R. Gibbons; Molecular and Cell Biology, University of California Berkeley, Berkeley, CA

The microtubule binding domain (MTBD) of dynein is separated from the AAA core of the motor by an ~15 nm stalk that is predicted to consist of an anti-parallel coiled coil. The structure of this coiled-coil and the mechanism by which it mediates communication between the MTBD and ATP-binding core are unknown. To identify the optimal alignment between the hydrophobic heptad repeats in the two strands of the stalk coiled-coil, we fused the MTBD of dynein, together with 12-36 residues (~25%) of its stalk, onto a stable coiled-coil base, provided by *Thermus thermophilus* seryl tRNA-synthetase (SRS). By assaying these chimeric constructs for microtubule binding in vitro, we identified an optimal register with 22 residues and 19 residues in the upstream and downstream strands of the coiled coil, respectively, (SRS-22:19) that yielded a protein possessing high-affinity (2.2 μM) for microtubules. Two approximately full-length stalk constructs with this same register of coiled coil strands (SRS-85:82 and SRS-99:96) showed a similarly high affinity at room temperature. Other register alignments generated by adding or deleting one or more

residues from the upstream strand of the full length constructs had ~10-fold lower affinity. This agrees with data from the SRS-22:19 construct and supports the hypothesis that dynein utilizes small amounts of sliding displacement between the two strands of its coiled-coil stalk as a means of communication between the AAA core of the motor and the MTBD. The binding of full-length constructs approximately doubled when the temperature was decreased from 37°C to 4°C, whereas that of SRS-22:19 was little changed, consistent with transitions between high and low affinity forms of the MTBD at physiological temperatures involving shifts in equilibrium between multiple allosteric forms of the full-length stalk.

985

**Dissection of Intra-molecular Communications between the ATPase Site and Microtubule-binding Site in the Dynein Heavy Chain**

T. Kon, K. Imamura, R. Ohkura, K. Sutoh; Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Tokyo, Japan

Cytoskeletal motor proteins utilize bidirectional intra-molecular communications between the track-binding site and ATPase site to exert their motile activities. The information on ATP hydrolysis at the ATPase site is transmitted to the track-binding site to modulate the track-binding affinity. Conversely, the status of track binding is communicated to the ATPase site to regulate the rate of the ATPase activity. Elucidation of the mechanism of these intra-molecular communications is a key to understanding how motor proteins exert motile actions along cytoskeletal tracks. As for dynein, a microtubule-based motor protein, its ATPase site(s) is located within the head domain containing six AAA+ modules, while the microtubule-binding site called stalk head is spatially separated from the head domain by a 10-15 nm slender stalk that is predicted to be formed from an antiparallel coiled coil. Therefore, the information at the ATPase site and microtubule binding site of dynein must be transmitted through the stalk coiled coil. However, the mechanism underlying the bidirectional communications remains unclear. To address this issue, we introduced mutations into the stalk head and/or stalk coiled coil of the dynein motor domain. The resultant mutant dyneins can be classified into two groups on the basis of their biochemical properties: the mutant dyneins in the first groups appeared to be trapped in an “inactivated state” showing ATPase activity at basal rate (5-15 $s^{-1}$); those in the second group appeared to be fixed in an “activated state” showing much higher ATPase rate (80-120 $s^{-1}$) that is comparable to the maximum rate of the microtubule-activated ATPase activity of wild type dynein. Based on these results, we propose that dynein utilizes small conformational changes at the stalk coiled coil to couple microtubule binding and ATPase activity.

986

**Characterization of Dynactin Complex in Budding Yeast**

J. Li, J. A. Cooper; Cell Biology and Physiology, Washington University in St. Louis, St. Louis, MO

Dynactin is a multi-subunit protein complex required for most dynein activities, proposed to serve either as a cargo adaptor or to enhance dynein motor processivity. Dynactin consists of two parts with eleven different polypeptides. 1) The actin-like Arp1 filament has conventional actin and capping protein at its barbed end and Arp11, p25, p27, and p62 at its pointed end. 2) The arm consists of $p150^{Glued}$, dynamitin, and p24/22. In *Saccharomyces cerevisae*, homologues of several dynactin subunits have been identified, and deletion of their genes produces phenotypes identical to those of dynein null mutations. On the other hand, homologues for p24/22, p25, p27 and p62 have not been identified, and the composition of yeast dynactin has not been defined biochemically. Here, we report the discovery of an apparent p24/22 homolog in budding yeast, based on the following: 1) The null mutant has a dynein-like phenotype for spindle movement. 2) The predicted polypeptide has an appropriate size and secondary structure. 3) The null mutant is synthetic with kar9 null but not dyn1 null. 4) The protein localizes to microtubule plus ends and the spindle pole body, as seen for Nip100 / $p150^{Glued}$. 5) Dynein accumulates of dynein at the plus end of microtubules in the null mutant, as seen with known dynactin mutants. Biochemical fractionations of intact dynactin complex are underway, which may identify other subunits of yeast dynactin. Based on our current understanding, dynactin is needed to offload dynein from the plus ends to the cortex.

987

**Dynein Supports Rapid Spindle Elongation in Anaphase B in the Fungus Ustilago maydis**

G. Fink,[1] J. Colombelli,[2] E. H. K. Stelzer,[2] G. Steinberg[1]; [1]Organismic Interaction, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany, [2]Light Microscopy Group, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

In animal cells spindle elongation in anaphase B is mediated by cytoplasmic dynein, which interacts with the cell cortex and is thought to exert pulling forces on astral microtubules (MTs). In contrast, in the model fungi Saccharomyces cerevisiae and Schizosaccharomyces pombe dynein has no obvious role in anaphase B. Here we describe the role and dynamic rearrangement of dynein in anaphase in Ustilago maydis. During the initial phase spindles elongate at ~1,23 µm/min and no dynein is found at the tips of astral MTs. When the spindle reaches a length of ~2 µm, spindle elongation rates increase to ~5,34 µm/min, which coincides with cortical sliding of astral MTs and rapid motion of the spindle. In conditional dynein mutants spindle initial elongation was normal, but neither cortical sliding of astral MTs, nor rapid elongation was found. Instead, spindles continued elongation at ~1,44 µm/min until they reach a length of ~9 µm, suggesting that dynein-independent forces mediate anaphase A, while anaphase B is driven by dynein. This notion is supported by the observation that dynein is absent from short spindles (up to ~2µm). Dynein appearance coincides with the formation of a single leading MT at both poles of the spindle that carries the strongest dynein signal which is reduced in dynamic instability. Cortical sliding of this MT goes with off-loading of plus-end located dynein at the cell cortex, suggesting that dynein-mediated cortical sliding exerts forces on the spindle. This notion is supported by the observation that a laser-induced cut of the spindle or the cleavage of the sliding MT results in rapid motility of both spindle-halves or the leading MT, respectively. Our data demonstrate that dynein provides the force for rapid spindle elongation in anaphase B in U. maydis.

988

***Tetrahymena thermophila* Dynein 2 Light Intermediate Chain (D2LIC) Contributes to but is not Essential for Intraflagellar Transport**

V. Rajagopalan,[1] D. E. Wilkes,[2] D. J. Asai[2]; [1]Department of Biological Sciences, Purdue University, West Lafayette, IN, [2]Department of Biology, Harvey Mudd College, Claremont, CA

Cells with cilia or flagella express two non-axonemal (“cytoplasmic”) dyneins, dynein-1 and dynein-2. Dynein-2 heavy chain (Dyh2) is required for retrograde intraflagellar transport (IFT). Dynein-2 is thought to be comprised of only two subunits, the Dyh2 heavy chain and the dynein-2 light intermediate chain (D2LIC). To understand the function of dynein-2 in *Tetrahymena*, we have characterized D2LIC. *Tetrahymena* expresses a single D2LIC gene and the predicted protein is 474 amino acids in length. *Tetrahymena* D2LIC shows 22% and 27% identity to D2LICs from

*Chlamydomonas* and human respectively. We have made knockout heterokaryons of D2LIC; the progeny of two heterokaryons completely lack the D2LIC gene in their somatic macronuclei (KO-D2LIC). KO-D2LIC cells have cilia but exhibit motility defects. KO-D2LIC cells have generation times 1.4 times longer, indicating compromised "rotokinesis", which is required for the completion of cytokinesis (Brown et al., 1999). KO-D2LIC cells swim 3.7 times slower than the wild type controls, and do not swim straight. Despite the presence of an oral apparatus, most of the KO-D2LIC cells fail to phagocytose fluorescent beads, indicating impaired ciliary activity. The KO-D2LIC culture changed with time. Early exconjugants included a significant number of multi-nucleated, very large "monster" cells. Several days later, these "monster" cells were far fewer. Early exconjugants had a high number of multiple micro- and macro-nuclei per cell; the proportion of abnormally nucleated cells decreased after several days. These results are consistent with *Tetrahymena* D2LIC being important but not essential for IFT. *Tetrahymena* D2LIC may serve to stabilize the dynein-2 heavy chains and thus increase the efficiency of retrograde IFT process. (Supported by grants from the National Science Foundation).

## Microtubule Dynamics & Assembly I (989-1010)

989

### Function of the GammaTuRC in Centrosomal and Chromatin-Mediated Microtubule Nucleation

J. Luders, T. Stearns; Biological Sciences, Stanford University, Stanford, CA

During Interphase the gamma-tubulin ring complex (gammaTuRC), the major microtubule nuleator in animal cells, is found at the centrosomes and in the cytoplasm. At the G2/M transition additional gamma-tubulin from the cytoplasm is recruited to the centrosomes. Microtubule nucleation by the gammaTuRC is important for the formation of the mitotic spindle and recruitment of gammaTuRC to mitotic centrosomes is most likely regulated by kinases. At metaphase the gammaTuRC localizes not only to the spindle poles but also to spindle microtubules. We have identified and characterized a new subunit of the human gammaTuRC which regulates gammaTuRC localization and is required for centrosomal and chromatin-mediated microtubule nucleation. Our data provides new insight into functions of the gammaTuRC other than its well established role in microtubule nucleation at the centrosome.

990

### Structural Properties and Microtubule-Nucleating Activity of Human γ-Tubulin

M. Moritz,[1] L. Rice,[2] K. Yonekura,[1] H. Aldaz,[1] M. Braunfeld,[2] T. Stearns,[3] D. A. Agard[2]; [1]Biochemistry & Biophysics, UCSF, San Francisco, CA, [2]Biochemistry & Biophysics, UCSF, HHMI, San Francisco, CA, [3]Biological Sciences, Stanford University, Stanford, CA

γ-Tubulin is the major component of the 2.2 MDa γ-tubulin ring complex, and is the key microtubule-nucleating protein in cells. Although structurally similar to α/β-tubulin, γ-tubulin exhibits very distinct oligomerization properties and can nucleate microtubules on its own in vitro from pure components. We are characterizing γ-tubulin's unique assembly properties and its interaction with α/β-tubulin using electron microscopy, helical reconstruction, and various hydrodynamic techniques. We have found that pure γ-tubulin exists as a monomer in 0.5M KCL, but in low salt it rapidly forms distinct oligomers ranging from tetramers to long filaments to sheets/tubes, depending on concentration, pH and the presence or absence of α/β-tubulin. Interestingly, unlike α/β-tubulin, γ-tubulin oligomerization does not depend on GTP or temperature. We are also exploring γ-tubulin's microtubule-nucleating activity in molecular detail using light-scattering assays and kinetic modeling. At low concentrations (60-100nM), γ-tubulin appears to accelerate the rate of nucleus formation rather than decreasing the nucleus size. The nucleation mechanism appears to change at a higher concentration of γ-tubulin (600-700nM), where it forms larger oligomers. This suggests that larger oligomers of γ-tubulin are more potent nucleators, apparently acting by stabilizing smaller oligomers of α/β-tubulin and thus decreasing the nucleus size. Oligomerization-dependent nucleation mechanisms should help us to understand the nucleation mechanism of the γ-tubulin ring complex, which likely contains 12 or 14 γ-tubulins.

991

### Structural Analysis of Recombinant Yeast Small Gamma-Tubulin Complex

A. Zelter,[1] L. M. Rice,[2] E. Muller,[1] B. Fox,[1] J. W. Kern,[1] D. A. Agard,[2] T. N. Davis[1]; [1]Department of Biochemistry, University of Washington, Seattle, WA, [2]Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, CA

Gamma-tubulin is required for nucleation of microtubules and spindle assembly in eukaryotic cells. It is found in two major complexes, a small complex (~11 S) and a large complex (~25 S). In yeast and other eukaryotic cells, the small complex contains two related proteins, (Spc97 and Spc98 in yeast) and gamma-tubulin (Tub4 in yeast). The stoichiometry is 2 Tub4 : 1 Spc97 : 1 Spc98 (Vinh et al., 2002). We expressed the yeast small complex in insect cells and analyzed the recombinant small complex by several methods. First, scanning transmission electron microscopy revealed triangular shaped structures of the approximate molecular mass expected for the small complex. Then the structure of the purified recombinant small Tub4-complex was determined to 25 Å resolution by single particle reconstruction of negative stained material. This higher resolution structure clearly revealed a Y-shaped complex. This is the first structure available for a small gamma-tubulin complex from any organism. The organization and orientation of the components in the Y is not known. Two gamma-tubulins modeled from the recent crystal structure of human gamma-tubulin (Aldaz et al., 2005) fit into the top of the Y structure. Since either Spc97 or Spc98 will form a complex with Tub4, one possibility is that Spc97 and Spc98 form the base of the Y with each one binding to one of the Tub4s in the arms. Vinh, D. B. N., J. W. Kern, W. Hancock, J. Howard and T. N. Davis (2002) Reconstitution and characterization of the budding yeast gamma-tubulin complex. Mol. Biol. Cell 13:1144-1157. Aldaz H., L. M. Rice, T. Stearns, D. A. Agard (2005) Insights into microtubule nucleation from the crystal structure of human gamma-tubulin. Nature 435:523-527.

992

### Insight into Microtubule Assembly from the Atomic Structure of Human Gamma-tubulin

L. M. Rice,[1] H. Aldaz,[2] T. Stearns,[3] D. A. Agard[1]; [1]Biochemistry, UC San Francisco, San Francisco, CA, [2]MCB, UC Berkeley, Berkeley, CA, [3]Biological Sciences, Stanford University, Stanford, CA

Microtubules are hollow polymers of αβ-tubulin that exhibit GTP-dependent assembly dynamics and comprise a critical part of the eukaryotic

cytoskeleton. Initiation of new microtubules *in vivo* requires γ-tubulin, organized as an oligomer within the γ-Tubulin Ring Complex (γ-TuRC) of higher eukaryotes. Structural insight into γ-tubulin, its oligomerization, and how it promotes microtubule assembly remains lacking. We have determined the 2.7Å crystal structure of human γ-tubulin bound to GTPγS. One of the crystal packing interactions recapitulates the lateral contacts between tubulins in the microtubule lattice and probably forms the basis for γ-tubulin oligomerization within the g-TuRC. Unexpectedly, γ-tubulin:GTPγS adopts a curved conformation very similar to that seen in GDP-bound microtubule depolymerization products. This suggests that, unlike signaling GTPases, tubulins may not undergo nucleotide-dependent conformational switching. To confirm the idea that nucleotide state does not directly regulate the conformation of αβ-tubulin, we demonstrated that allocolchicine binds αβ-tubulin:GTP and αβ-tubulin:GDP with essentially identical affinity. These results have important implications for αβ-tubulin assembly; in particular, they suggest that there are unanticipated barriers to *de novo* microtubule assembly. We propose a model for microtubule assembly in which guanine nucleotides do not regulate curved-to-straight transitions, but instead serve to modulate the strength of longitudinal interactions within the microtubule lattice.

993

**Multiple Mechanisms for Microtubule Generation and Pole Focusing of the Mitotic Spindle**

G. Goshima,[1] N. M. Mahoney,[1] F. Nedelec,[2] R. D. Vale[1]; [1]Cellular and Molecular Pharmacology, UCSF, San Francisco, CA, [2]EMBL, Heidelberg, Germany

During the formation of metaphase spindle in animal somatic cells, microtubules (MTs) are generated from centrosomes (C-MTs) and chromosomes. These two MT networks need to be interconnected and focused by minus-end-directed motor proteins to create the diamond-shaped appearance of the bipolar spindle. We have characterized the roles of two minus-end-directed motors, dynein and Ncd, in such processes in Drosophila S2 cells using RNAi and high resolution microscopy. While the motors have overlapping functions, we show that Ncd is primarily responsible for focusing kinetochore microtubule bundles (K-fibers), while dynein has dominant function in transporting the K-fibers to the centrosomes. We also report a novel localization of Ncd to the growing tips of C-MTs, which we show is mediated by the plus-end-tracking protein EB1. Computer modeling of the pole focusing process suggests that the plus end localization of Ncd could facilitate the capture and transport of K-fibers along C-MTs. From these results and simulations, we propose a model on how two minus-end-directed motors cooperate to ensure spindle pole coalescence during mitosis. We also investigated the mitotic function of 28 genes implicated in centrosome function in S2 cells and identified several proteins as yielding pronounced mitotic spindle defects after RNAi depletion. Live cell imaging of GFP-tubulin demonstrated that γ-tubulin is required for generation of both C-MTs and chromosomal MTs. By tracking analysis of EB1-GFP, we also uncovered another process of MT formation originating from spindle microtubules possibly in a γ-tubulin independent manner. We speculate that such de novo MT growth may involve a novel mechanism of templating from pre-existing MTs.

994

**Mechanisms for Mitotic Spindle Pole Focusing in the Absence of Functional Centrosomes**

G. Goshima, A. Kirby, H. Barak, L. Krueger, S. Sivaramakrishnan, R. D. Vale; Physiology Course 2005, Marine Biological Laboratory, Woods Hole, MA

The centrosome is the dominant microtubule (MT) nucleation site during mitotic spindle formation. However, animal somatic cells also can generate MTs near chromosomes independently of centrosome function. In Drosophila S2 cells, RNAi of centrosomin (CNN) leads to loss of functional centrosomes, and spindles MTs are generated and organized into a bipolar array via chromatin-dependent pathway, as occurs during meiosis. Despite the absence of functional centrosomes, the spindle MTs, including kinetochore microtubule bundles (K-fiber), are reasonably well focused at their minus-ends in CNN RNAi cells. To understand the mechanism of K-fiber focusing in the absence of centrosomes, we performed double RNAi screening of CNN with ~200 known mitotic genes, and identified Ncd (a minus-end-directed kinesin) and Asp (a putative homolog of vertebrate NuMA) as essential factors for K-fiber focusing in CNN spindle. Interestingly, unlike what has been described for meiotic spindles reconstituted using Xenopus egg extracts, RNAi knockdown of dynein/dynactin subunits did not affect the pole focusing of acentrosomal spindles in S2 cells. We suggest that pole focusing by dynein requires a centrosome and involves to transport K-fibers along centrosome MTs. Ncd and Asp, on the other hand, can crossbridge minus ends of K-fibers and contribute to pole focusing in the absence of centrosomes.

995

**Microtubule Flux in Early Mitosis**

N. P. Ferenz, P. Wadsworth; Biology, University of Massachusetts, Amherst, MA

To date, the study of microtubule flux during mitosis has been limited to metaphase and anaphase. Because kinetochore microtubules attach to chromosomes prior to metaphase, we considered the possibility that these fibers may flux during this time. Using a permanent mammalian cell line expressing α-tubulin tagged with a photoactivatable variant of GFP as well as the inner centromere protein CenpA tagged with GFP (LLCPK1-PA-CenpA), we have observed poleward movement of photoactivated regions during prometaphase. Additionally, we have detected similar motions in the centrosomal array of microtubules prior to nuclear envelope breakdown (NEB). These data suggest 1) that microtubule flux may contribute to mitosis in ways previously undetected and 2) a mechanism for kinetochore fiber flux that might differ from the established model, as the antiparallel microtubules necessary for the proposed actions of Eg5 may be unavailable in developing spindles. In order to determine the mechanism responsible for promoting flux at these early stages, photoactivation is being used to analyze flux following siRNA of candidate molecules (Kif2a, Eg5 and MCAK). Although a fully sequenced pig genome is yet to be completed, a large collection of pig ESTs can be found on the NCBI database, including partial sequences for the above listed molecules. The pig sequences for Kif2a and Eg5 are identical in the regions used for siRNA of human Kif2a and Eg5, and siRNA of pig Eg5 generates a monopolar phenotype. Thus, we can deplete candidate genes and directly observe microtubule behavior during spindle formation. Our observations suggest that mitotic microtubules undergo poleward flux during prophase and prometaphase in mammalian cells.

996

**Examining the Function of Microtubule Localized RNAs during Mitosis**

M. D. Blower, K. Weis, R. Heald; Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Accurate segregation of chromosomes during mitosis and meiosis is required for organismal development and viability, and depends on the assembly and function of a dynamic mitotic spindle that orchestrates chromosome movements. Previous work has demonstrated that several proteins involved in RNA metabolism (Rae1, Maskin, and CPEB) are localized to the mitotic spindle and contribute to spindle assembly. Furthermore, we have shown that RNA itself is localized to mitotic microtubules in *Xenopus* egg extracts, and contributes to spindle assembly in a translation independent manner that may reflect a structural role. We have found that several different types of microtubule-associated RNA are present on the spindle including ribosomal RNA and messenger RNA (MT-mRNA). We have used Affymetrix gene chip microarrays to determine which mRNAs are specifically enriched on the mitotic spindle. While translation of RNA is not required for spindle assembly in egg extracts, our preliminary results suggest that translation of a subset of MT-mRNAs contributes to mitotic progression, while a distinct subset of MT-mRNAs are found in inactive storage granules likely to function later in development. We will present evidence that translation of a key cell cycle regulator is required for exit from a Spindle Assembly Checkpoint (SAC) induced mitotic arrest, suggesting that mitotic translation is regulating anaphase onset. We will also present the results of experiments investigating how MT-mRNAs are targeted to the spindle, and the functional importance of spindle-localized protein translation. These experiments should provide insight into the role of MT-mRNAs during mitosis and development.

997

**Microtubules and Interphase Cell Cycle Progression**

Y. Uetake, G. Sluder; UMass Medical School, Worcester, MA

The importance of cytoplasmic microtubules for interphase cell cycle progression in mammalian somatic cells has been unclear due to the diversity of published experimental results. We use normal human primary fibroblasts and hTERT-RPE1 cells to systematically investigate the involvement of cytoplasmic microtubules in interphase cell cycle progression. We affect a complete and rapid disassembly of microtubules at anaphase-telophase by treating mitotic shake off cells for 10 min. at 0 °C followed by continuous exposure to 3.2 uM nocodazole. We find that the cells complete mitosis and progress through interphase with normal kinetics into the next mitosis, at which point they arrest due to microtubule absence. In separate experiments we quantitatively examine the relationship between prolongation of mitosis and subsequent G1 progression. We prolong mitosis with low dose (0.08 uM) nocodazole that leave small bipolar spindles or disorganized spindles. Upon release from nocodazole the cells divide in a normal bipolar fashion regardless of the mitotic durations examined. If mitosis is prolonged less than ~45 minutes, the cells progress through interphase into the next mitosis with normal kinetics. If the block is longer than ~1 hour, the cells arrest in G1 for at least 48 hr. Similar results were obtained using 100 uM monastrol to prolong mitosis by preventing spindle bipolarization. Prolongation of mitosis by less than 1 hour allows the cells progress through interphase into the next mitosis with normal kinetics while longer delays lead to a G1 arrest. We conclude that cytoplasmic microtubules have no required function for complete interphase progression in normal, p53 competent human cells. However, modest prolongation of mitosis in the presence of spindle microtubules leads to a G1 arrest once the cells complete an otherwise normal division.

998

**Microtubule and Myosin II Dynamics during Cytokinesis in Echinoderm Embryos**

V. E. Foe; Center for Cell Dynamics, University of Washington, Friday Harbor, WA

Recent studies suggest a positive role for stable microtubules in furrow induction (Shannon et al., 2005; Canman et al., 2003). By treating echinoderm blastomeres for five minutes with 20 uM nocodazole (over 100X the concentration needed to depolymerize most of this cell's microtubules) we have revealed a previously undescribed transient population of stable microtubules, which begins forming at anaphase onset (first near chromosomes), grows out in all directions as anaphase proceeds, by telophase converts into an array roughly coextensive with what is commonly called the "central spindle", and disappears as cell cleavage completes. The time of their formation, their localization, and the finding by others that taxol-stabilized microtubules can induce furrowing (Shannon et al., 2005) leads us to suspect that this nocodazole-resistant array is involved in signaling equatorial RhoA (and myosin II) activation. Using fixed echinoderm blastomeres and an antibody that recognizes the activating phospho-serine19 of RLC we show that activated myosin II (a small fraction of all myosin II) localizes to the cortex, waxing and waning in phase with the cell cycle. Upon metaphase exit there is a brief large-scale increase in phospho-serine19 RLC over the entire cell cortex. Then as dynamic elongating astral microtubules reach the cortex in anaphase/telophase (initially before stable microtubules make contact) there ensues a progressive global diminution of activated cortical myosin II, possibly because microtubules remove it or microtubule polymerization modulates Rho signaling. Nocodazole treatment during early anaphase that eliminates dynamic astral microtubules before cortical phospho-serine19 MRLC diminution, inhibits or slows furrowing and either leaves the whole cell surface myosin-enriched or widens the contractile furrow. Our findings suggest a mechanism whereby dynamic microtubule outgrowth induces polar relaxation (except that relaxation is global not just polar) while stable microtubules signal myosin recruitment to the furrow.

999

**G Protein-coupled Receptors Activation Induces Gβγ Translocation and Promotes Microtubule Assembly in NIH3T3 Cells**

C. Gutierrez, S. Das, S. Roychowdhury; Biological sciences, University of Texas at El Paso, El Paso, TX

βγ subunit of G proteins is known to transfer signals from cell surface receptors to intracellular effector molecules. Recent genetic analyses of *C. elegans* and *Drosophila* reveal that Gβγ is also involved in regulating mitotic spindles. Previously we have shown that βγ subunit of G proteins promotes microtubule assembly in vitro [Roychowdhury and Rasenick (1997*) J. Biol. Chem.* 272, 31476-31581]. Gβγ was found to interact preferentially with microtubules (rather than tubulin dimers) in both NIH3T3 and PC12 cells. Using microtubule-acting drugs, Gβγ was shown to be involved in microtubule assembly. To further elucidate the possible link between G protein activation and microtubule modulation by Gβγ, NIH3T3 cells were treated with isoproterenol, an agonist for β-adrenergic receptors (cell surface receptors linked to Gs) and UK14,304, an agonist for α2- adrenergic receptors (cell surface receptors linked to Gi) for 1h. After agonist treatment, cytoskeletal fraction (enriched in microtubules) and soluble protein fraction (enriched in tubulin dimer) were isolated. Gβγ association with microtubules and soluble tubulin fractions as well as the changes in microtubule assembly was determined. Both isoproterenol and UK14,304 increased the Gβγ association with microtubule fraction by ~75%. In addition, promotion of microtubule assembly and/or stabilization of microtubules was observed in the presence of both agonists (~50%). Since activation of both Gs and Gi produced similar effects, the possible involvement of cAMP in this process was ruled out. Our result suggests that G protein-coupled receptors are involved in the modulation of microtubules by mobilizing Gβγ to bind to tubulin/microtubules.

1000

**CLASP-Dependent Nucleation Contributes to the Asymmetry of Microtubule Arrays**

A. Kharitonov,[1] N. Andreeva,[1] A. Efimov,[2] A. Khodjakov,[3] N. Galjart,[4] J. V. Small,[1] A. Akhmanova,[4] I. Kaverina[2]; [1]IMBA, Vienna, Austria, [2]Vanderbilt University Medical Center, Nashville, TN, [3]Wadsworth Center, Albany, NY, [4]Erasmus University, Rotterdam, The Netherlands

Microtubules differentially regulate the front and the rear of motile cells due to the dynamic asymmetry of their system. As microtubule nucleation at the centrosome is symmetric, other mechanisms are responsible for differential changes in microtubule system. CLASPs are microtubule tip-binding proteins that also localize to centrosome and Golgi apparatus, sites capable of the microtubule nucleation and anchoring. We studied the role of CLASPs in microtubule nucleation at Golgi complexes and in the microtubule pattern. We analyzed microtubule plus ends dynamics in the cell center by video imaging of GFP-EB3 expressing motile cells. Microtubule outgrowth started at centrosome or Golgi membranes. While centrosomal nucleation was symmetric, Golgi-associated microtubule array was not. Further, we used siRNA approach to investigate the role of CLASP in microtubule nucleation. CLASP depletion blocked Golgi-associated microtubule outgrowth. Dense microtubule meshwork, characteristic for Golgi region, is absent in CLASP-depleted cells, indicating disturbed microtubule-Golgi association. Microtubule re-growth assay upon nocodazole washout confirmed that CLASPs were essential for initiation of microtubules at Golgi. Our data indicate that CLASPs participate in nucleation and/or stabilization of microtubule minus ends at Golgi membranes. We propose that this novel CLASP function is essential for the asymmetry of microtubule arrays in motile cells.

1001

**The Fission Yeast CLASP Homolog Promotes Stability of Interphase Microtubules**

S. V. Bratman, F. Chang; Microbiology, Columbia University, New York, NY

CLIP-associated proteins (CLASPs) constitute a family of microtubule-associated proteins conserved from yeasts to mammals. Recent studies have suggested roles for CLASPs in regulating microtubule dynamics at kinetochores in mitosis and at the cell cortex in interphase. Here we characterize the single CLASP homolog in the fission yeast *Schizosaccharomyces pombe.* CLASP overexpression produces a novel phenotype on interphase microtubule dynamics. Microtubules are properly organized and extend the length of the cell, but appear to be excessively stable and bundled. In contrast to normal microtubules, which rarely display rescue, in cells overexpressing CLASP, microtubules grow and shrink in only very small excursions at the cell tips, indicative of increased microtubule rescue frequency. These effects on microtubule plus-end dynamics are independent of tip1p (CLIP170), mal3p (EB1) and mto1p (γ-TuC). CLASP may also stabilize MT minus-ends, as treadmilling microtubules in the γ-TuC mutant *mto1Δ* become less dynamic upon overexpression of CLASP. When expressed at endogenous levels, CLASP localizes primarily to peri-nuclear dots on interphase microtubules and to regions of the mitotic spindle, although when overexpressed, it decorates entire microtubules. Analyses of loss-of-function mutants will also be presented. These studies suggest that fission yeast CLASP may play a direct role in the regulation of microtubule stability.

1002

**The Role of β-catenin/APC Interactions in Microtubule Dynamics**

R. K. Louie, S. Bahmanyar, W. J. Nelson, A. I. M. Barth; Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA

β-catenin and the adenomatous polyposis coli (APC) tumor suppressor protein are important components in the Wnt signal transduction pathway. In this pathway, β-catenin stability is regulated by the state of phosphorylation by the kinase GSK-3β. Mutations in these proteins are implicated in cancers, including breast and colorectal carcinomas. In addition to the Wnt pathway, APC also plays a role in microtubule organization and cell migration. We have begun to explore structural roles of β-catenin binding to APC in the regulation of microtubules in cells expressing mutant β-catenin that cannot be phosphorylated by GSK-3β. This stabilized form of β-catenin localizes prominently to APC clusters at the tips of membrane extensions. Live cell imaging of GFP-tubulin reveals a decrease in the dynamic instability of microtubules in cells expressing this stabilized β-catenin. Microtubules in these cells spend more time in pause, and are noticeably less dynamic at the cell edge. Current work aims at elucidating the mechanism by which β-catenin binding to APC regulates the microtubule cytoskeleton.

1003

**Apc Protein Modulates Local Microtubule Assembly/disassembly Dynamics Independently of Eb1**

K. Kita,[1] T. Wittmann,[1] I. S. Näthke,[2] C. M. Waterman-Storer[1]; [1]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Division of Cell & Developmental Biology, University of Dundee, Dundee, United Kingdom

Regional regulation of microtubule (MT) assembly/disassembly dynamics is critical to polarized cell behaviors such as directional migration, neuronal pathfinding, and the immune response. In interphase cells, the adenomatous polyposis coli (APC) protein accumulates on a small subset of MTs in cell protrusions. This implies that APC may be involved in regulating the assembly/disassembly dynamics of these specific MTs. To test this hypothesis, we used a fluorescently labeled monoclonal antibody that recognizes an N-terminal APC epitope to act as a non-perturbing probe to visualize dynamics of endogenous APC and MTs labeled with a spectrally distinct fluorophore in living cells. Analysis of APC-decorated and non-APC-decorated MT plus ends revealed that MTs decorated with APC spent more time growing and had a decreased catastrophe frequency than neighboring MTs in the same cell not decorated with APC. We also made the novel discovery that endogenous APC could associate with shortening MTs. To determine the relationship between APC and its binding partner EB1, we monitored EB1-GFP and endogenous APC concomitantly in living cells. Only a small fraction of APC co-localized with EB1 at any one time. Using APC-deficient cell lines and siRNA of EB1, we found that EB1and APC localized at MT ends independently. Depletion of EB1 did not change the growth-stabilizing effects of APC decoration on MT plus ends. In addition, EB1 immediately dissociated from MT plus ends after cells were treated with submicromolar concentrations of nocodazole, while APC did not. Our observations demonstrate for the first time that the association of endogenous APC protein correlates directly with increased MT growth stability independent of its association with EB1, and show that APC and EB1 associate with MT plus ends by distinct mechanisms.

1004

**Molecular Dynamics Imaging in Micro-patterned Living Cells**

K. Kandere-Grzybowska,[1] C. Campbell,[2] Y. Komarova,[1] O. Chaga,[1] B. Grzybowski,[2] G. G. Borisy[1]; [1]Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL, [2]Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL

A hallmark of cancer metastasis is activation of cell motility machinery leading to cell movement and invasion. Generating cells of uniform shape and predictable cytoskeletal organization by substratum micro-patterning techniques would enable quantitative analysis of cell motility subcomponent processes. Current micro-patterning approaches using self-assembled monolayers of alkyl thiols on gold are incompatible with important imaging modalities in cell biology because of absorption of light and scattering of electrons by gold. Here, we describe a novel procedure--Anisotropic Solid Microetching (ASOMIC)--that overcomes these limitations. ASOMIC employs reaction-diffusion processes in patterned agarose stamp to microetch a uniform gold layer that is subsequently rendered anti-adhesive. The method allows molecular dynamics imaging by wide-field and total internal reflection fluorescence (TIRF) microscopy of living cells and correlative platinum replica electron microscopy. Microtubule (MT) dynamics was investigated in B16F1 mouse melanoma cells from time-lapse series of yellow-fluorescent protein fusion with MT plus-end cytoplasmic linker protein-170 (YFP-CLIP170). Upon impact with cell edge, MTs often turned and continued to grow along it. While cell shape had no significant effect on MT growth velocity or growth persistence, “edge impact” turning behavior was dependent on the shape of cell edge--MTs turned more frequently when encountering straight edges of triangular cells, rather than convex perimeters of circular cells. Initial results with triangular cells suggest a pathway for guidance of MT growth. This method allows for high-resolution dynamic imaging of fluorescently-labeled molecules in geometrically-defined, living cells and is compatible with other important imaging modalities. It is likely to be of broad applicability to cell biology, especially to understanding control mechanism of directional cell motility and cancer metastasis. Supported by Grant GM 25062 (GGB), Camille and Henry Dreyfus New Faculty Award (BG), and DOD Breast Cancer Research Program postdoctoral fellowship (KKG).

1005

**In Vitro Reconstitution with CLIP-170, EB1 and p150$^{Glued}$ Demonstrates Distinct Mechanisms of Microtubule Plus-End Tracking**

L. A. Ligon, J. L. Ross, M. K. Tokito, E. L. F. Holzbaur; Department of Physiology, University of Pennsylvania, Philadelphia, PA

Microtubules in the cell are highly dynamic, and microtubule plus-end proteins are important regulators of this behavior. Plus-end proteins dynamically track the tips of growing microtubules *in vivo*, but the mechanisms driving this specific localization are not well understood. Here we used binding experiments, *in vitro* reconstitution, and total internal reflection (TIRF) microscopy with the purified tip-tracking proteins CLIP-170, p150$^{Glued}$, and EB1 to test potential mechanisms for plus-end specific localization. Binding experiments demonstrate that CLIP-170 binds tubulin dimers with an affinity several-fold higher than that for polymerized microtubules. In contrast, p150$^{Glued}$ binds to microtubules with a higher affinity than to tubulin dimers. Under these experimental conditions, EB1 binds weakly to both tubulin and microtubules. *In vitro* reconstitution assays show that CLIP-170 can co-polymerize with tubulin, and polymerization assays with TIRF microscopy demonstrate that CLIP-170 is dynamically associated with microtubule ends. p150$^{Glued}$ and EB1, on the other hand, do not co-polymerize with tubulin, suggesting that they utilize different mechanisms for the robust *in vivo* plus-end localization they demonstrate. Binding experiments show that EB1 can bind to CLIP-170. Further, CLIP-170 can recruit EB1 to tubulin dimers and this complex co-polymerizes with tubulin, suggesting that EB1 may be localized to plus-ends via its association with other plus-end proteins. We have previously shown that EB1 binds directly to p150$^{Glued}$ (Ligon *et al*., Mol Biol Cell, 2003), and here we show that CLIP-170 and p150$^{Glued}$ compete for binding to EB1. These results suggest that there may be multiple microtubule plus-end complexes and these complexes may differentially regulate microtubule dynamics. *Supported by NIH GM48661.*

1006

**Actin-dependent Regulation of Microtubule Dynamics via Interaction between mDia1 Formin and Alpha-tubulin Deacetylase HDAC6**

C. Ballestrem,[1] Y. Zilberman,[2] L. Carramusa,[2] B. Gilquin,[3] M. Shtutman,[2] S. Khochbin,[3] A. D. Bershadsky[2]; [1]Faculty of Life Sciences, University of Manchester, Manchester UK, United Kingdom, [2]Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel, [3]Faculte de Medecine, Institut Albert Bonniot, La Tronche Cedex, France

This study reveals a link between actin cytoskeleton and microtubules mediated by interaction between mDia1 formin and alpha-tubulin deacetylase HDAC6. Expression of a constitutively active form of formin mDia1 not only enhanced actin polymerization but also triggered profound alterations in microtubule dynamics. Measurements of microtubule plus end growth using GFP/RFP-fusion constructs of microtubule end-tracking proteins or alpha-tubulin revealed that active mDia1 decreased the microtubule elongation rate by a half, irrespective of which marker for the growing microtubule was used. This reduction of microtubule growth rate was abolished by treatment with inhibitors of actin polymerization, suggesting that the effect of mDia1 on microtubule dynamics is mediated by actin filaments. We then found a more direct relationship between mDia1 and microtubules. In latrunculin B-treated cells, micron-sized patches containing active mDia1 moved rapidly along microtubules. In cells expressing active mDia1, the fraction of microtubules containing acetylated alpha-tubulin increased dramatically. Since alpha-tubulin acetylation is controlled by its specific deacetylase, HDAC6, we determined whether this enzyme is involved in the mDia1-induced alteration of microtubule dynamics. Co-immunoprecipitation experiments demonstrated that HDAC6 associated with mDia, and both mDia1 and HDAC6 further interact with the plus-end tracking proteins EB1 and Arp1. Blocking HDAC6's deacetylase activity by tubacin or TSA affected microtubule dynamics in the same way as active mDia1. HDAC6-siRNA knockdown experiments showed that HDAC6 is necessary for mDia1 to decrease microtubule growth, while overexpressing wild-type, but not catalytically inactive, HDAC6 partially restored microtubule growth in mDia1-expressing cells. Thus, HDAC6 with inhibited deacetylase function mediates mDia1's effect on microtubules. We conclude that mDia1 and HDAC6 are constituents of a molecular complex responsible for a dynamic link between growing ends of microtubules and actin filaments.

1007

**The Formin mDia Stabilizes Microtubules Independently of Its Actin Nucleation Activity**

F. Bartolini,[1] J. B. Moseley,[2] J. Schmoranzer,[1] B. L. Goode,[2] G. G. Gundersen[1]; [1]Pathology & Cell Biology, Columbia University, New York, NY, [2]Biology, Brandeis University, Waltham, MA

A major microtubule (MT) polarization event in directed cell migration is the Rho-mDia dependent stabilization of a subset of MTs oriented

towards the direction of migration (Palazzo et al., NCB, 2001; Wen et al., NCB, 2004). The stabilization induced by Rho-mDia in vivo is achieved by capping of MT plus ends making them refractory to tubulin subunit exchange. mDia and other formins nucleate actin filaments, yet, it is unclear whether this activity or a separate activity of mDia is involved in MT stabilization. Based upon the crystal structure of the yeast formin Bni1, we prepared two mutants (K853A and I704A) in a constitutively active version of mDia2 and found that these mutants were unable to promote efficient actin nucleation in vitro, to induce actin filaments or filopodia in vivo or to "surf" at the ends of actin filaments in vivo. Strikingly, both these mutants still retained the ability to induce the formation of stable MTs in serum-starved cells and to bind to the MT TIP proteins EB1 and APC. A dimerization mutant of mDia2 (W630A) was also inactive toward actin but retained the ability to generate stable MTs in vivo. These results show that promotion of actin assembly and induction of MT stability by mDia can be functionally separated. In agreement with this, recombinant wild type mDia1, mDia2 and actin nucleation mutants of mDia2 all bound directly to MTs in vitro. Also, the minimally active fragment of mDia2 (containing just FH1 and FH2 domains) inhibited assembly of MTs from tubulin subunits and stabilized assembled MTs from depolymerization in vitro. These results show that the formin mDia has a direct activity to stabilize MTs, perhaps by capping dynamic MT ends.

1008

**Contrasting Actions of SCG10 and Stathmin on Microtubule Dynamic Instability at Steady State in vitro**

T. Manna,[1] D. Thrower,[1] G. Grenningloh,[2] L. Wilson[1]; [1]Molecular, Cellular, Developmental Biology, University of California Santa Barbara, Santa Barbara, CA, [2]Centre de Neurosciences Psychiatriques, University Hospital of Lausanne, Lausanne, Switzerland

Stathmin and SCG10, members of the stathmin family of microtubule (MT) destabilizing proteins, are highly expressed in the developing nervous system. SCG10 is believed to regulate growth cone MT dynamics in response to extracellular signals during axonal growth. While several studies have examined the effects of stathmin on dynamic instability, little is known about how other stathmin family members regulate MT dynamics. We have analyzed the effects of SCG10 (35-179) on dynamic instability *in vitro* and find that its actions are distinct from those of stathmin. At steady state, conditions in which the MT polymer and tubulin concentrations remain constant, stathmin strongly and selectively destabilizes minus ends by increasing the catastrophe frequency. It has little or no effect on the rate or extent of growth or shortening at either end. We further find that stathmin increases the MT treadmilling rate and that it binds to MTs *in vitro* along their lengths. SCG10 regulates dynamic instability differently than stathmin. The major effect of SCG10 at plus ends is to increase the growth rate and the length grown during growth events. Like stathmin, SCG10, increases the plus end catastrophe frequency, but much more weakly than stathmin. The major effect of SCG10 at minus ends is to increase the shortening rate. Like stathmin, SCG10 also increases the minus end catastrophe frequency, but more weakly than stathmin. Like stathmin, SCG10 also binds to MTs along their lengths. The ability of SCG10 to increase plus end growth is consistent with its postulated role in neurite outgrowth (Riederer et al., *Proc. Natl. Acad. Sci.* **94**:741-45, 1997). The results also indicate that an important cellular action of both stathmin and SCG10 may be to destabilize MT minus ends. Supported by NS13560.

1009

**Study of the Function of Wild-type Op18/Stathmin and Mutant Q18→E in Microtubule Polymerization and Dynamics**

H. A. Wilson, D. A. Thrower, L. Wilson; Molecular, Cellular and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA

Stathmin/Op18 is a microtubule destabilizing, ubiquitous, cytosolic protein that is highly expressed in neuronal cells. Much debate has occurred and continues, concerning the actual mechanism utilized by stathmin to inhibit the formation of microtubules by inducing the catastrophe/depolymerization phase in microtubule dynamics. The mechanisms proposed include a "Sequestering "model in which Op18 forms a T2S (tubulin-stathmin-tubulin) complex with tubulin subunits, thus preventing polymerization from occurring, and also a "Binding" model in which Op18 directly binds to microtubule structures to induce depolymerization. While our recent experimental findings are consistent with the both of the proposed mechanisms, we also observed that stathmin is capable of inducing catastrophes independently of sequestration *in vitro*, which we confirmed with a sequestration deficient mutant. A recent study discovered a mutant form of stathmin (Q18→E) in an esophageal adenocarcinoma that possesses the ability to transform normal cells and promote tumor formation. We observed a significantly reduced effect on microtubule polymerization by the Q18→E mutant relative to wild-type stathmin. Another recent study indicated that stathmin deficient mice developed an age-dependent axonopathy of the peripheral and central nervous systems. Our goal is to further understand the role of stathmin in microtubule dynamics by comparing the ability of this mutant and wild-type stathmin to bind to tubulin, to undergo phosphorylation, in addition to a comparison of their effects on microtubule dynamics. Clarification of stathmin's role in microtubule dynamics would facilitate our understanding of its role in diseases in which its expression is elevated such as in various cancers, demyelinating disorders such as multiple sclerosis, and age-related disorders.

1010

**XMAP215 Inhibits the Localization of XKCM1 to Microtubule Ends**

J. H. Stear, G. J. Brouhard, J. Howard, A. A. Hyman; Max-Planck Institute for Cell Biology and Genetics, Dresden, Germany

The *X. laevis* protein XKCM1 belongs to an evolutionarily conserved family of kinesin-like proteins which function in vivo and in vitro as potent microtubule destabilizers. These proteins exhibit a high affinity for microtubule ends, where they catalyze the removal of tubulin subunits and promote catastrophe events. The catastrophe promoting activity of XKCM1 is opposed by XMAP215, a microtubule associated protein that localizes along the length of the microtubule. It is thought that the balance between the activities of these two proteins plays a central role in determining the steady state length of microtubules within a cell. However, it remains unclear how XMAP215 antagonizes the activity of XKCM1. We have developed an assay, using total internal fluorescence microscopy (TIRF), to examine the real-time interactions of recombinant XKCM1-GFP with GMPCPP stabilized microtubules. Confirming previous data, we observe that XKCM1 is highly enriched at microtubule ends. In the presence of XMAP215, this end-binding is abolished, suggesting a potential mechanism for how XMAP215 antagonizes the microtubule destabilizing activity of XKCM1. To explore this question further, we examined the behavior of single XKCM1-GFP molecules while bound to microtubules and observed that XKCM1 undergoes random, 1D diffusion along the microtubule lattice. Similar behavior similar has been seen with MCAK, the human homologue of XKCM1, and it has been proposed that this motion provides a mechanism for the rapid targeting of XKCM1 to microtubule ends. We are currently testing the hypothesis that XMAP215 blocks the diffusion of XKCM1 along the lattice, which

inhibits its ability to localize to microtubule ends, and antagonizes its catastrophe promoting activity.

## Cilia & Flagella I (1011-1036)

1011

### Localization of EB1, IFT Polypeptides, and Kinesin II in Chlamydomonas Flagellar Axonemes via Scanning Electron Microscopy

R. D. Sloboda,[1] L. Howard[2]; [1]Department of Biological Sciences, Dartmouth College, Hanover, NH, [2]Dartmouth Electron Microscope Facility, Dartmouth College, Hanover, NH

We are using an FEI scanning electron microscope equipped with a thermionic field emission gun (FEG-SEM) to co-localize in the flagellar axoneme different pairs of polypeptides double-labeled with specific antibodies. This approach provides a new perspective on the morphology of the particles involved in intraflagellar transport (IFT), their association with the axoneme, and their protein composition. The FEG-SEM as configured delivers a resolution of ~5 nm with biological samples when operated at 15 kV. Flagella were isolated from *Chlamydomonas* and extracted with 0.1% NP-40 at room temperature. The resulting axonemes were reacted in solution with various primary antibodies specific for flagellar proteins, washed by sedimentation, and then reacted with gold-labeled secondary antibodies. The labeled axonemes were attached to polyethyleneimine coated coverslips and finally fixed in 1% glutaraldehyde. The samples were then critical point dried, coated with 2-3 nm of osmium using a plasma coater, and imaged at 15 kV. Detection of backscattered electrons (BSE) clearly identifies both 12 and 25 nm gold particles while detection of secondary electrons (SE) provides a conventional SEM view of the labeled axonemes; comparison of the BSE and SE images reveals the location of the gold, and hence the antigens, relative to overall axonemal morphology. *Chlamydomonas* EB1 localizes to the plus ends of the outer doublet microtubules, as expected from previous immunofluorescent data (Pedersen et al. Curr. Biol. 13:1969-74.). IFT particles can be identified that are double labeled with antibodies to either kinesin II and IFT 139 (a complex A polypeptide) or kinesin II and IFT 172 (a complex B polypeptide). Experiments are in progress to determine if single individual IFT particles identifiable by FEG-SEM are composed of both complex A and complex B polypeptides. Supported by NSF MCB-0418877.

1012

### Gli2 and Gli3 Localize to Cilia and Require the Intraflagellar Protein Polaris for Function

C. J. Haycraft, B. Banizs, B. K. Yoder; Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL

Cilia are projections extending from the surface of many eukaryotic cells that can be either motile or immotile and are constructed through a process termed intraflagellar transport (IFT). *Tg737* encodes an IFT protein required for cilia formation that when disrupted in $Tg737^{\Delta 2\text{-}3\beta\text{-}gal}$ mutants results in 8-10 unpatterned digits on the limbs. The lack of ectopic *Shh* expression in $Tg737^{\Delta 2\text{-}3\beta\text{-}gal}$ mutants along with the formation of multiple non-patterned digits is reminiscent of the phenotype seen in $Shh^{-/-};Gli3^{-/-}$ double mutant embryos and recent data has shown a genetic link between IFT/polaris and *Shh* signaling. In agreement with this data, we find that primary cells isolated from the limbs of $Tg737^{\Delta 2\text{-}3\beta\text{-}gal}$ mutant embryos are non-responsive to exogenous ShhN suggesting that *Tg737* is required for *Shh* pathway activation in the responding cell. Exogenously expressed Gli2 and full length Gli3 are nonfunctional in mutant cells while Gli3 repressor and Gli1 function are unaffected. Additionally, we detect an increase in the relative amount of unprocessed Gli3 in $Tg737^{\Delta 2\text{-}3\beta\text{-}gal}$ mutants. Together these data suggest that IFT/polaris is required upstream of Gli transcriptional activity, likely at the level of Gli processing. Furthermore, our data also suggest a direct role for cilia in this signaling pathway as all three Gli proteins and Sufu, a negative regulator of Gli function and part of the complex involved in Gli processing, localize to the distal tip of cilia in primary limb bud cultures. We propose that the distal tip of the cilia functions as a site for concentrating components of the hedgehog pathway for regulation by proteolytic processing. In the absence of cilia, these proteins are diffusely located and are processed inefficiently resulting in the non-responsive nature of the mutant cells.

1013

### Functional Genomic Analysis of Sensory Cilia from *C elegans*

O. E. Blacque, C. Li, P. N. Inglis, M. R. Leroux; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

*C. elegans* is a useful model for elucidating the molecular basis of cilia function and cilia-related diseases such as polycystic kidney disease and Bardet-Biedl syndrome (BBS). For example, we previously showed that the *C. elegans* BBS proteins play important roles in intraflagellar transport (IFT) - a kinesin and dynein driven motility that builds and maintains cilia - by mediating the functional coordination of the two kinesin-2 motors that drive anterograde IFT (Blacque et al., Genes Dev. 18: 1630-1642; Ou et al., Nature 436: 583-587). Specifically, we found that in *bbs* mutants, IFT rafts are destabilized, resulting in the separation of the two kinesin-2 motors and IFT complexes A and B. To identify novel cilia-related components, in particular those associated with BBS and IFT gene pathways, we recently employed functional genomics in *C. elegans* and found many candidate ciliary genes, including a novel IFT component, DYF-13 (Blacque et al., Curr. Biol. 15: 935-941). To extend this work, we have now utilized microarray technology to identify gene targets of the ciliogenic RFX transcription factor, DAF-19. By comparing the expression profiles of wild-type and *daf-19* mutant worms, we identified new candidate cilia-related genes, including a novel conserved IFT-associated gene, which we name IFTA-1. We subsequently generated a deletion allele of *ifta-1* and found that these animals display structural and functional ciliary defects, including a severely abrogated IFT process. Interestingly, *ifta-1* mutant phenotypes are similar to those of *che-11* mutants, suggesting that IFTA-1 is an IFT complex A type protein. Using *bbs* mutants as tools for destabilizing the IFT raft, we are currently employing genetics and fluorescence microscopy to further characterize IFTA-1 function. Furthermore, these approaches may be useful for establishing a genetic model for the assembly of the entire motor-IFT particle complex.

1014

### Intraflagellar Transport Protein IFT27 is a Rab-Like Small G Protein that Functions in the Cell Cycle

H. Qin, Z. Wang, J. L. Rosenbaum; MCDB Dept., Yale University, New Haven, CT

Intraflagellar transport (IFT) is a bi-directional motility system occurring in most eukaryotic flagella/cilia. IFT is required for flagellar formation and maintenance, but whether it has a role in other cellular functions is not known. IFT particles contain multiple proteins forming two complexes, A and B. Here, we report that IFT27, a 27 kD component of IFT complex B, is a Rab-like small G protein. In *Chlamydomonas*, GFP tagged IFT27

moves bi-directionally inside the flagellum. Like other previously-reported IFT particle proteins, IFT27 is also concentrated at the basal body region. However, unlike other IFT proteins, a fraction of IFT27 from *cell body extracts* does not sediment at 16S with the rest of complex B, but rather remains at the top of the sucrose density gradient. In *flagellar* extracts all the IFT27 sediments at 16 S. Surprisingly, when the level of IFT27 protein is knocked down by RNAi, cells no longer divide normally. In such RNAi knock down cells, flagella can be assembled and maintained but multiple basal body sets and nuclei are observed and the cells become very large. The cells often fail to complete mitosis and eventually die. Mutations affecting other *Chlamydomonas* IFT particle proteins result only in flagellar defects; knock down of IFT27 is unique in having an effect on cell division. Currently we are using over-expression, constitutive active and dominant negative forms of IFT27 to further understand how IFT27 functions in the cell cycle. This is one of the first examples of a *bona fide* ciliary protein, IFT27, having a cell cycle regulatory function. (Supported by NIH grant14642 to JLR.)

1015

**Kin5 Knockdown in Tetrahymena Using RNAi**

A. Awan, A. Bell, P. Satir; Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY

In previous studies [2004: Cell Motil 58:1-9], we demonstrated that Kin5, a homodimeric Kinesin-2 in *Tetrahymena*, is a ciliary protein that showed colocalization with orthologs of *Chlamydomonas* IFT complex proteins along the cilia. Kin5 is an intraciliary transport (ICT) motor distinct from the ICT kinesins, Kin1 and Kin2. In this study, we examined the effect of knocking down Kin5 on ciliogenesis, cell motility and survival, and transport of a putative cargo protein. We devised a modification the standard knockout method to yield a somatic mutant, so that RNA interference (RNAi) can be induced in *Tetrahymena.* We demonstrate that an RNAi construct for *KIN5* has been stably integrated into the genome, such that with appropriate induction, while control proteins are unaffected, Kin5p is severely downregulated and disappears from the cilia. Because Kin5 is an essential component, cell motility decreases by 95.3% and knockdown is lethal by 12 h. However, unlike Kin1p/Kin2p knockout, the Kin5p knockdown does not cause ciliary resorption, while deciliation in the knockdown condition reveals that ciliary axonemes assemble and remain motile in the absence of ciliary Kin5p. Since axonemal construction seems unaffected, we examined whether specific intraciliary non-axonemal cargos might be affected by the knockdown. We chose a protein, Gef1, related to yeast Sec7, a guanine nucleotide exchange factor, that is found in ciliary membrane-matrix fractions and that localizes along the cilium. Gef1 coimmunoprecipitates with Kin5. Nevertheless, Gef1 remains in the cilium under conditions where ciliary Kin5 disappears. Upon deciliation, Gef1 immunofluorescence is absent in the regenerating cilia of the knockout cells. This suggests that Gef1 and similar cargos could be transported into the cilium by the Kin5 complex, and then released into a position along the cilium, where they could act in signal transduction pathways to affect motility and/or viability.

1016

**Dissecting the Molecular Mechanisms of Intraflagellar Transport**

L. B. Pedersen,[1] S. Geimer,[2] J. L. Rosenbaum[3]; [1]Molecular Biology and Physiology, University of Copenhagen, Copenhagen, Denmark, [2]Biology and Electronmicroscopy, University of Bayreuth, Bayreuth, Germany, [3]MCDB Department, Yale University, New Haven, CT

Intraflagellar transport (IFT) is a bi-directional microtubule-based transport system essential for the assembly and maintenance of eukaryotic cilia/flagella. In *Chlamydomonas*, the IFT system consists of two motor complexes, kinesin-2 and cytoplasmic dynein 1b (cDynein1b), and an IFT particle containing ca. 17 different polypeptides in two complexes, A and B. Kinesin-2 contains two motor subunits, FLA10 and FLA8, and one non-motor subunit, KAP. cDynein1b contains heavy chain (HC) and light intermediate chain (LIC) subunits, and possibly additional as yet unidentified subunits. Although analysis of mutants with defects in various components of the IFT system have provided significant insights into the molecular mechanisms of IFT, several questions remain unsolved. In the current model for IFT, kinesin-2 carries cDynein1b, IFT particles and axonemal precursors from the basal body to the flagellar tip, and cDynein1b transports kinesin-2, IFT particles and axonemal turnover products back to the cell body. The mechanisms by which cargo unloading/loading, and IFT motor exchange occur at the flagellar tip are unknown. We have used a variety of *Chlamydomonas* mutants with defects in kinesin-2 (KAP, FLA10), cDynein1b (LIC), or IFT complex B (IFT172) and RNAi to begin to dissect the molecular mechanisms of IFT. We find that kinesin-2 can associate with cDynein1b HC independently of LIC and IFT complex A and B, and show that binding of cDynein1b to kinesin-2 requires KAP. Furthermore, we show by transmission EM that the tip turn-around point for IFT particles is localized distal to the +end of the B outer doublet microtubules on which IFT occurs. Based on these and previous findings we propose a new model for IFT in which tip-turnaround involves 1) dissociation of complex A and B, 2) binding of A to cDynein1b LIC, 3) re-association of B with A prior to retrograde IFT.

1017

**Role of IFT46 Protein in Intraflagellar Transport (IFT)**

Y. Hou,[1] H. Qin,[2] W. L. Dentler,[3] G. J. Pazour,[4] J. L. Rosenbaum,[2] G. B. Witman[1]; [1]Cell Biology, UMASS Medical School, Worcester, MA, [2]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, [3]Division of Biological Sciences, University of Kansas, Lawrence, KS, [4]Program in Molecular Medicine, UMASS Medical School, Worcester, MA

IFT, the bi-directional movement of particles along the length of eukaryotic cilia and flagella, is required for ciliary and flagellar assembly and maintenance. The IFT machinery includes IFT-particle proteins and anterograde and retrograde motors. Biochemical analysis of *Chlamydomonas* IFT particles showed that they contain ~16 proteins, arranged into complexes A and B. We have cloned IFT46, a putative complex B protein. Quantitative RT-PCR shows that IFT46 expression is induced upon deflagellation. Immunofluorescence microscopy (IFM) shows that IFT46 has a cellular localization typical for IFT-particle proteins. We identified a *Chlamydomonas* null mutant for IFT46. Western blotting indicates that this mutant has elevated levels of complex A proteins and reduced levels of complex B proteins, with the exception of IFT172, which is near wild-type level. Whereas other published IFT-particle protein mutants are aflagellate, *ift46* has short, stumpy flagella. IMF indicates that the flagella contain some IFT-particle proteins, but IFT particles are not detected by EM. EM also reveals that the axonemes lack outer arms and have defects in central pair assembly. This is in contrast to mutants in the retrograde IFT motor, DHC1b, which have short, stumpy flagella that accumulate IFT particles and can form a normal axonemal structure. The defects are rescued by the wild-type IFT46 gene, confirming that they are due to lack of IFT46. Thus, IFT46 is important for the stability of complex B and for transporting cargo, including the outer arms, into flagella. We also found that IFT46 is phosphorylated and identified 3 phosphorylation sites by MS analysis. Two constructs that encode modified IFT46 genes were transformed into

the null mutant. The transformants express modified IFT46 that mimics either the phosphorylated or non-phosphorylated form. Further characterization of these transformants is underway to determine the function of IFT46 phosphorylation.

1018

**Bardet-Biedl Syndrome 3 is a Centrosomal Small GTP-Binding Protein Associated with Intraflagellar Transport**

M. A. Esmail, M. Kang, M. R. Leroux; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

Bardet-Biedl syndrome (BBS) is a pleiotropic disorder linked to eight genes and typified by abdominal obesity, retinal degeneration, polycystic kidneys, polydactyly, anosmia and cognitive impairment. Recent work with *M. musculus* and *C. elegans* mutants has pointed to a defect in cilia as the underlying cause of BBS. Current evidence suggests a transport or trafficking function for BBS proteins. We have recently shown that mammalian BBS4 likely functions in the retrograde transport of pericentriolar material 1 protein, which is required for anchoring microtubules at the centrosome. We have also demonstrated that *C. elegans* BBS7 and BBS8 participate in intraflagellar transport (IFT), a process required to build functional sensory cilia. Using a bioinformatic approach in conjunction with DNA sequence analysis, we discovered that *ARL6* is the gene responsible for the last uncloned *BBS* gene, *BBS3*. *ARL6/BBS3* encodes an ADP-ribosylation factor-like protein, belonging to the Arf/Arl family of small GTP-binding proteins. Immunocytochemistry reveals that ARL6/BBS3 localizes to the centrosome in IMCD-3 cells, a ciliated mouse kidney cell line. This localization occurs independently of microtubules as neither nocodazole nor over expression of p50 dynamitin affect the centrosomal signal. In *C. elegans*, ARL6/BBS3 is found at the base of cilia and undergoes IFT. 3D modeling of ARL6 indicates that all the substitution mutations identified in human patients map to the GTP-binding pocket of ARL6, and GTP-binding assays confirm that each of the protein mutants have disrupted nucleotide binding. Although Arf/Arl proteins had previously been implicated in intracellular transport, our findings show that at least one member is associated with IFT and is one of several genes causing BBS, a disorder that affects a large number of vital systems in humans.

1019

**LF4p, a Regulator of Flagellar Length in Chlamydomonas, is a Cargo for Transport by IFT**

N. F. Wilson, P. A. Lefebvre; Plant Biology, University of Minnesota, St. Paul,, MN

Chlamydomonas actively maintains its two flagella at an equal and appropriate length. Genetic studies have identified four genes (*LF1, LF2, LF3*, and *LF4*) that when mutated generate cells with abnormally long flagella. *LF4* was recently shown to encode a MAP kinase with a unique C-terminal domain. Both the kinase activity and the C-terminal domain of LF4p are required for rescue of the long-flagella phenotype. In cell bodies LF4p migrates as a doublet at 72/73 kDa, whereas LF4p in flagella migrates as a single band at 65 kDA. The shift of LF4p from a single band in flagella to a doublet in cell bodies was confirmed by 2D SDS-PAGE. Both cell body forms of LF4p are phosphorylated on the TEY motif suggesting that in cell bodies LF4p is an active kinase. The flagellar form of LF4p is maintained in a largely inactive state by the activity of one or more phosphatases. Analysis of *lf4* flagella revealed an increase in the level of FLA10p. Consistent with an interaction between LF4p and FLA10p, LF4p was lost from *fla10* flagella at the restrictive temperature. Sucrose gradient fractionation of flagella revealed that LF4p co-fractionated with IFT particle proteins at 16S. LF4p, however, did not co-fractionate with either complex A or complex B proteins under high salt conditions. Interestingly, the inclusion of phosphatase inhibitors during high salt extraction of flagella prevented the dissocation of complex A and complex B suggesting they interact via one or more phosphorylated proteins. Moreover, in the presence of phosphatase inhibitors, FLA10p co-fractionated with IFT particles at 16S suggesting that this interaction is also dependent on phosphorylation. Experiments are currently underway to identify the phosphatase(s) that regulates the kinase activity of LF4p in flagella.

1020

**Architectural Analysis of the *Chlamydomonas* Intraflagellar Transport Complex B**

B. F. Lucker,[1] R. H. Behal,[1] H. Qin,[2] J. L. Rosenbaum,[2] D. G. Cole[1]; [1]Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID, [2]Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT

Intraflagellar transport (IFT) is a strongly conserved process required for the assembly and function of eukaryotic cilia and flagella. During IFT, large protein particles carry axonemal precursors to the distal tip of the organelles while axonemal turnover products are ferried back to the cell body (Qin *et al.*, 2004). Excluding motor complexes, IFT particles consist of multiple copies of two separate complexes, A (at least 6 subunits) and B (at least 13 subunits). Here we report on our ongoing characterization of the *Chlamydomonas* complex B architecture. We have found that several B subunits dissociate from the complex in moderate ionic strength (~300 mM NaCl) revealing a salt-stable core that contains IFT88, IFT81, IFT74, IFT72, IFT52, IFT46 and IFT27. Using yeast-based two-hybrid analysis, we have identified a 200 amino acid coiled-coil region of IFT74/72 that interacts with the coiled-coil subunit, IFT81. Similar analysis reveals that IFT81 has the ability to homodimerize. Using a combination of biochemical and yeast-based three-hybrid analyses we have found IFT81, IFT74, and IFT72 appear to form a higher order oligomer within the complex B core at a ratio of 2:1:1, respectively. Currently we are using additional approaches, including chemical cross-linking, to further characterize these and other complex B interactions. Supported by GM61920 (DGC), P20RR016454 (DGC) and GM14642 (JLR).

1021

**Proteomic Investigation of the Eukaryotic Flagellum Using the Model Organism *Trypanosoma brucei***

R. Broadhead,[1] H. Dawe,[2] S. Hart,[3] N. Portman,[2] M. Ginger,[2] S. Gaskell,[3] P. McKean,[1] K. Gull[2]; [1]Department of Biological Sciences, Lancaster University, Lancaster, United Kingdom, [2]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, [3]Michael Barber Centre for Mass Spectrometry, University of Manchester, Manchester, United Kingdom

We have used the newly completed *T. brucei* genome project along with mass spectrometric techniques to investigate the proteomic constituents of a eukaryotic flagellum. Salt-extracted axonemes were isolated from *T. brucei* strain 427 and subjected to one-dimensional or two-dimensional SDS-PAGE. Bands, spots and regions were excised, trypsin-digested, and analysed by LC-MS/MS. Mascot was used to search the *T. brucei* genome for the most likely peptide matches. 522 proteins were identified. 128 were discarded following verification of mass spectra by manual sequencing, leaving 394 proteins. We used the presence of ribosomal proteins in this dataset as a marker for contamination by highly basic proteins aberrantly binding to tubulin following disruption of cell membranes. Discarding proteins with a predicted pI of greater than 10.1 left a final dataset of 341 proteins that we confidently predict to be genuine components of the salt-extracted flagellum. These data were further interrogated by

comparative genomics using an informative set of model organisms. Proteins were separated by their phylogenetic distribution into trypanosomatid-specific, flagellate-specific, and those with a distribution not wholly restricted to flagellates. Approximately 50% of the dataset fell into the first group and, as the roles of cilia and flagella vary widely both between and within organisms, these probably represent organism-specific structures and functions. Two-thirds of the remaining proteins were flagellate-specific, representing conserved components of the axoneme. The remaining proteins include tubulins and calcium-binding proteins that have cellular roles in addition to their function in flagella. Finally, we have compared our dataset to other bioinformatic and proteomic studies and generated a list of 134 proteins that have appeared in multiple studies. We propose that this cohort of proteins forms a core axoneme conserved across eukarya.

1022

**Insights into Flagellar Function and Disease from RNAi Validation of a Flagellar Proteome**

H. Dawe,[1] H. Farr,[1] N. Portman,[1] P. McKean,[2] K. Gull[1]; [1]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, [2]Department of Biological Sciences, Lancaster University, Lancaster, United Kingdom

We have recently completed a proteomic and comparative bioinformatic analysis of the *Trypanosoma brucei* flagellum. In order to validate our proteome biologically, a set of proteins from our final dataset was selected for investigation by RNAi. This set covers each of the major groups identified in our bioinformatic screen (trypanosomatid-specific; flagellate-specific; and those with a distribution not wholly restricted to flagellates). Protein expression was knocked down by inducible RNAi and the mutant cell lines were scored for aberrant flagellar, growth and cell morphogenesis phenotypes. Induction of RNAi did not prevent flagellum assembly for any of the proteins examined; however we found motility phenotypes consistent with a defect in the flagellum. Two proteins did not produce a phenotype on RNAi induction, and proved to be members of a gene family in *T. brucei*. We therefore performed simultaneous ablation of both proteins and observed a slow growth phenotype and paralysis of the flagellum, indicating gene redundancy. We analysed our proteomic dataset for the presence of *T. brucei* orthologues of known and putative ciliary disease genes. We identified the orthologues of 11 genes known to cause diseases with a clinical spectrum suggestive of ciliary dysfunction. Four of these represent novel axonemal components that we identify in this work. In addition over 45 proteins in our set have orthologues that map to genetic loci linked to diseases that have pathologies consistent with ciliary defects, and represent a valuable resource for the identification of novel ciliary disease genes.

1023

**IC97, A Novel Dynein Intermediate Chain From Flagellar Inner Arm Dynein I1, Interacts with Tubulin In Situ**

M. Wirschell,[1] T. W. Hendrickson,[2] L. Fox,[1] N. A. Haas,[3] C. D. Silflow,[3] G. B. Witman,[4] W. S. Sale[1]; [1]Cell Biology, Emory University School of Medicine, Atlanta, GA, [2]Department of Biology, Morehouse College, Atlanta, GA, [3]Department of Plant Biology, University of Minnesota, St. Paul, MN, [4]Department of Cell Biology, University of Massachusetts, Worcester, MA

Our goal is to determine the molecular basis of assembly and regulation of flagellar dyneins, particularly *Chlamydomonas* inner arm dynein I1. Here we focus on a previously uncharacterized I1 intermediate chain (IC), IC97. Sequences of peptides from band-purified IC97 were determined by MS/MS and used to clone the IC97 gene, which maps to linkage group XV. IC97 is a novel IC without notable structural domains such as WD-repeats. BLAST reveals IC97 shares homology to an axonemal protein in *Ciona* and with Las1 proteins - encoded by part of the multigenic pulmonary adenoma susceptibility 1 (Pas1) locus implicated in lung tumorigenesis in mice. An IC97 antibody detects a ~90-kDa protein in purified I1 fractions and isolated axonemes; this protein is missing in I1-defective mutants (see also Bower *et al*., these proceedings). Previous data determined an axonemal protein, postulated to be IC97, interacts with IC138 and IC140 (Hendrickson *et al*., 2004 and Yang *et al.* 1998). To test this, EDC crosslinking was used to identify IC97-interacting proteins. No bands corresponding to the IC138 and IC140 crosslinked products were detected. Rather, a ~140-kDa crosslinked product was identified, indicating IC97 interacts with a ~50-kDa axonemal protein. The crosslinked product was immuno-precipitated and components identified by MS/MS. In addition to IC97, the only other significant peptides obtained corresponded to α- and β-tubulin. The interaction of IC97 and α-tubulin was confirmed in EDC-crosslinked axonemes from an HA-tagged α1-tubulin strain. These results demonstrate that IC97 interacts with tubulin components *in situ*, and suggests that IC97 is located at the base of I1, providing additional evidence for the localization of ICs at the doublet microtubule "cargo" binding site.

1024

**The Flagellar A-Kinase Anchoring Protein, RSP3, is a Dimer**

M. Wirschell,[1] F. Zhao,[1] D. Diener,[2] A. R. Gaillard,[3] C. Yang,[4] P. Yang,[4] J. Rosenbaum,[2] W. S. Sale[1]; [1]Cell Biology, Emory University School of Medicine, Atlanta, GA, [2]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, [3]Deparment of Biology, Sam Houston State University, Huntsville, TX, [4]Department of Biology, Marquette University, Milwaukee, WI

Experimental evidence in *Chlamydomonas* has demonstrated that the radial spokes are required for regulation of dynein-driven microtubule sliding and control of ciliary/flagellar bend symmetry. In particular, radial spoke protein 3, RSP3, is an A-kinase anchoring protein thought to anchor PKA and locally regulate dynein. To further explore the role of RSP3, we used EDC crosslinking to identify RSP3 interacting proteins. EDC crosslinking in isolated axonemes results in 150-kDa RSP3 product. Several independent experiments demonstrate that the 150-kDa crosslinked product is a dimer of RSP3. First, the extracted crosslinked product co-sedimented with isolated 20S radial spokes and was also formed in the isolated 20S radial spokes and 12S radial spoke precursor. Therefore, the crosslinked product was strictly formed within individual radial spokes, between radial spoke proteins. Second, given the size of the crosslinked product, RSP3 interacts with a second RSP3 or another similar sized radial spoke protein. To distinguish between these possibilities, two approaches were taken; crosslinking of mutant axonemes missing specific spoke proteins, and 2D gel analysis of the crosslinked product's pI. The 150-kDa RSP3 crosslinked product was successfully formed in mutant axonemes lacking the spoke head, RSP2 or RSP23. 2-D gels demonstrate the pI of the crosslinked product is the same as un-crosslinked RSP3. Third, the 150-kDa crosslinked product was formed using purified, bacterially expressed RSP3. Fourth, native gel electrophoresis demonstrated the purified recombinant RSP3 migrated as two distinct species; a faster migrating form that co-migrated with denatured, monomeric RSP3, and a slower migrating form-indicative of an RSP3 dimer. These data support a model in which each radial spoke is founded on an RSP3 homodimer, and indicate that each radial spoke can localize two PKAs in position to control dynein activity and axonemal bending.

1025

**Stepwise Ciliary Growth on Sea Urchin Embryos Supported by Kinesin-2**

R. L. Morris, D. Erkoboni, J. Nordberg, C. N. English, F. J. Dufort, A. Manning; Biology, Wheaton College, Norton, MA

The ciliary lifecycle in sea urchin embryos involves the assembly, maintenance, retraction and disassembly of the axoneme at least once each mitotic cell cycle. Previous evidence suggests that cilia on sea urchin embryos first form an assembly intermediate, or procilium, that requires the activity of kinesin-2 to mature into a full-length beating organelle. To test this hypothesis, we employed quantitative light microscopy and GFP-tagged kinesin-2 subunits to investigate patterns of cilia growth in sea urchin embryos and the roles of kinesin-2 in the ciliary lifecycle. Although ciliary elongation was steady when cilia growth was monitored as a population, pauses in cilia growth during ciliogenesis were detected in both *L. pictus* and *L. variegatus* embryos when the growth of individual cilia was monitored. Pauses in growth occurred consistently in the range of lengths predicted for procilia. Final lengths of *L. variegatus* cilia fell into three discrete modes of 2 μm, 11 μm, and 23 μm suggesting that stepwise ciliary maturation may occur over successive cell divisions. Interestingly, sister cells (twin daughters of the same mother cell mitosis) on *L. variegatus* embryos were observed repeatedly and synchronously growing and retracting cilia in the 11 μm length mode without undergoing mitosis. Ciliary retractions were typically continuous and linear and showed that retraction acts to pull the axoneme down and out of its plasma membrane sheath possibly using motors at the ciliary base. Roles of kinesin-II in the stepwise assembly and maturation of cilia was investigated using the GFP-tagged non-motor KAP subunit of kinesin-2. Observations of KAP-GFP redistributing between basal bodies, axonemes, and nuclei support the hypothesis that kinesin-2 in sea urchin embryos plays roles in ciliary assembly and maintenance through intra-flagellar transport (IFT) and may play a signaling role in the nucleus.

1026

**A Molecular Dissection of Ciliogenesis**

E. S. Kannegaard, W. F. Marshall; Biochemistry, University of California San Francisco, San Francisco, CA

Cilia are known to be crucial for many developmental and physiological processes, yet the establishment and maintenance of cilia are not well understood. In particular, apart from intraflagellar transport proteins, molecular players in the process of ciliogenesis are largely unknown. Two recent studies utilizing the unicellular green alga *Chlamydomonas reinhardtii*, which has two flagella that are homologous to human cilia, have determined the proteome of the flagellum and identified genes that are upregulated upon deflagellation. By analyzing these datasets we have identified a set of genes that are upregulated during flagellar regeneration for which the gene products are not components of the flagellum itself, suggesting that there is a class of proteins whose function is to act as cofactors in flagellar assembly. Potential roles for these proteins include chaperones, signaling intermediates, and targeting proteins. We propose to investigate the functions of ciliogenesis cofactor proteins utilizing knockdown and knockout strategies in *Chlamydomonas* and ciliated mammalian tissue culture cells.

1027

**Identification and Characterization of a Guanine Nucleotide Exchange Factor in *Tetrahymena thermophila***

A. J. Bell, C. Guerra, A. Awan, P. Satir; Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

In 1999, a guanine nucleotide exchange factor (GEF) was identified in *Paramecium* from a screen for ciliary proteins and was named PSec7 (Nair et al, FASEB J.) Use of the *Paramecium* PSec7 antibody in *Tetrahymena thermophila* recognized a putative GEF protein, Gef1p, which localizes to the cilia in immunofluorescence, immunoEM and western blot experiments. Gef1p coimmunoprecipitates with and is probably transported by the *Tetrahymena* intraciliary transport kinesin motor protein, Kin5. PCR methods and database analysis were used to identify *GEF*1. A protein 2053 amino acids in length, containing Sec7 motifs, truncated IQ motifs and PH domains, homologous to similar domains in Psec7, was cloned. Upregulation of the product of this gene was observed via RT-PCR following deciliation and subsequent regrowth of cilia in *Tetrahymena* cells. In order to confirm previous experimental results for Gef1p, a second antibody is being made against the cloned protein. In addition, a knockdown of the cloned protein will be performed by utilizing RNAi. A proposed model for the role of GEFs in the cilium will be presented based on these data.

1028

**A Lis1-like Protein is Associated with Axonemal Outer Arm Dynein in *Chlamydomonas***

L. B. Pedersen,[1] J. L. Rosenbaum,[2] S. M. King[3]; [1]Molecular Biology and Physiology, University of Copenhagen, Copenhagen, Denmark, [2]MCDB Department, Yale University, New Haven, CT, [3]Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, Farmington, CT

Lissencephaly is a severe developmental brain disorder characterized by a smooth cerebral surface, thickened cortex, and misplaced neurons. Type I lissencephaly is caused by mutations in the *LIS1* gene, which encodes a WD-repeat protein previously implicated in cytoplasmic dynein regulation, nuclear migration, and mitosis (Morris et al., 1998, Trends Cell Biol., 8:467-470). Several proteins involved in nuclear migration in *Aspergillus* bind directly to Lis1, including the nuclear movement protein NudC (Morris et al., 1998, Curr. Biol., 8:603-606). Mammalian NudC is highly expressed in ciliated epithelia, and localizes to motile cilia in various tissues, including brain (Gocke et al., 2000, Histochem. Cell Biol., 114:293-301). Moreover, a NudC ortholog is upregulated upon deflagellation in *Chlamydomonas* (Stolc et al., 2005, PNAS, 102:3703-3707). We cloned a gene encoding a Lis1-like protein (CrLis1) from *Chlamydomonas*. CrLis1 contains 347 residues with a mass of 37.2 kDa and contains seven WD-repeat domains, similar to Lis1 proteins from other organisms. Immunoblotting using an antibody raised against CrLis1 revealed that this protein is present in flagella and is mostly solubilized following detergent treatment. Furthermore, CrLis1 is depleted from flagella of mutants with defects in outer arm dynein assembly (*oda1*, *oda3*, *oda6*), but is present in strains missing other axonemal structures including inner arms (*ida1*, *ida4*), radial spokes (*pf14*) and the central pair microtubule complex (*pf18*). GST pull-down experiments indicate that CrLis1 physically interacts with outer arm dynein components from *Chlamydomonas* flagella. Furthermore, CrLis1 binds directly to rat NudC indicating that it is a functional ortholog of the mammalian Lis1 protein. These results suggest that CrLis1 and NudC are present in cilia/flagella and may play a role in regulating outer arm dynein activity.

1029

**Analysis of a Rab-like Protein Involved in Lifespan Regulation in *C elegans* Cilia**

J. C. Schafer,[1] B. DuBose,[1] C. J. Haycraft,[1] P. Swoboda,[2] B. K. Yoder[1]; [1]Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [2]Department of Biosciences, Karolinska Institute, Section of Natural Sciences, Södertörn Univerity College, Huddinge, Sweden

Cilia are important structures that function in a variety of ways including sensory perception, cell and fluid motility, and embryonic patterning. Intraflagellar Transport (IFT) is a mechanism by which proteins necessary for cilia formation and maintenance are assembled into large rafts at the base of the cilia and are carried along the cilium axoneme in anterograde and retrograde directions by motor proteins. Several IFT genes in *C. elegans* are regulated by the transcription factor DAF-19 which binds to a sequence known as an X-box in the promoters of these genes. Based on this fact, we have identified a novel cilia protein, T28F3.6, that shares significant homology with members of the Rab family of proteins involved in protein trafficking. T28F3.6 is homologous to the RabL5 protein that is conserved among ciliated eukaryotes including human, mouse, and *Drosophila*. We have found that T28F3.6::GFP localizes to cilia in *C. elegans* and moves along the cilium axoneme similar to other IFT proteins. Furthermore, mutation of a single amino acid believed to be involved in GTP binding causes delocalization of the protein throughout the ciliated sensory neurons. Also, analysis of T28F3.6::GFP in several known cilia mutants shows that T28F3.6::GFP remains localized to cilia despite loss of other IFT proteins. Characterization of two mutants in the T28F3.6 gene in *C. elegans* has shown an extended lifespan phenotype. Interestingly, these mutants show no defects in cilia morphology and also do not exhibit other cilia mutant phenotypes such as osmotic avoidance or chemotaxis defects indicating that T28F3.6 is not a typical IFT protein involved in ciliogenesis. Therefore, we are continuing our research into the role of T28F3.6 in the *C. elegans* lifespan pathway as a way to discern its role in the cilia.

1030

**The *C elegans* Homologs of Nephrocystin-1 and Nephrocystin-4 are Cilia Transition Zone Proteins Involved in Chemosensory Perception**

M. E. Winkelbauer,[1] J. C. Schafer,[1] C. J. Haycraft,[1] P. Swoboda,[2] B. K. Yoder[1]; [1]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [2]Department of Biosciences, Section of Natural Sciences, Karolinska Institute, Sodertorn University College, Huddinge, Sweden

Nephronophthisis (NPH) is a cystic kidney disorder that causes end-stage renal failure in children. Five nephrocystin (nephrocystin-1 to nephrocystin-5) genes whose function is disrupted in NPH patients have been identified and data indicate they form a complex at cell junctions and focal adhesions. More recently, the nephrocystin proteins have also been localized to cilia, as have multiple other cystic kidney disease related proteins. Significant insights into this cilia and cystic kidney disease connection have come from analyses in simpler eukaryotic organisms such as *C. elegans*. In this regard, we became interested in the *C. elegans* homologs of nephrocystin-1 (*nph-1*) and nephrocystin-4 (*nph-4*) due to an in silico based screen to identify genes coordinately regulated by the ciliogenic transcription factor DAF-19. Here we show that expression of *nph-1* and *nph-4* is DAF-19 dependent, that their expression is restricted to ciliated sensory neurons, and that both NPH-1 and NPH-4 concentrate at the transition zones at the base of the cilia, but are not found in the cilia axoneme. In addition, NPH-4 is required for the localization of NPH-1 to this domain. Interestingly, *nph-1* or *nph-4* mutants have no obvious cilia assembly defects; however, they do have abnormalities in cilia mediated sensory functions as evidenced by abnormal chemotaxis and lifespan regulation. Our data suggest that rather than having a ciliogenic role, the NPH proteins play an important function as part of the sensory or signaling machinery of this organelle. These findings suggest that the defects in human NPH patients may not be the result of aberrant ciliogenesis but abnormal cilia-sensory functions.

1031

**Epitope Tagging of Novel, Conserved Flagellar Proteins in *Chlamydomonas reinhardtii***

K. F. Lechtreck, G. B. Witman; Depratment of Cell Biology, University of Massachusetts Medical School, Worcester, MA

Proteomic analysis of flagella isolated from *Chlamydomonas reinhardtii* has identified more than 600 proteins by two or more peptides (Pazour et al. 2005, JCB 170:103). This data set includes known proteins that were not previously reported to be flagellar constituents, and numerous previously uncharacterized proteins, of which ~90 are highly conserved in vertebrates. We are using epitope tagging to test whether the latter are genuine flagellar proteins, and to determine their locations within the flagellum. To date, 16 genes have been cloned by PCR and ligated into a vector that fuses three consecutive HA tags to the C-terminus of the gene products. Eight of these (C_20334, C_30068, C_320063, C_380055, C_410061, C_450085, C_630058, C_650031), with predicted products in a size range from 36 to 90 kDa, were transformed into Chlamydomonas and stably expressed as revealed by western blotting using anti-HA. Indirect immunofluorescence of methanol-fixed cells confirmed a flagellar localization for all eight; five fusion proteins showed additional localization at the basal bodies or in the cytoplasm. Within the flagellum, distinctly different distributions of the fusion proteins were observed, including preferential labeling of the subdistal part, exclusion from the distal part, and a proximo-distal decrease in signal strength. Six of the eight proteins could be detected after detergent extraction and aldehyde fixation, suggesting a firm association with the axoneme. The results show that HA-tagging is a suitable method for rapid validation of putative flagellar proteins. HA-tagging also should facilitate identification of those components that interact with the tagged proteins.

1032

**Proteins of the Radial Spoke, a Mechanochemical Signal Transducer in 9+2 Cilia and Flagella**

P. Yang,[1] D. R. Diener,[2] C. Yang,[1] T. Kohno,[3] G. J. Pazour,[4] J. M. Dienes,[1] N. Agrin,[5] S. M. King,[6] W. S. Sale,[7] R. Kamiya,[3] J. L. Rosenbaum,[2] G. B. Witman[5]; [1]Biology, Marquette Universty, Milwaukee, WI, [2]MCDB, Yale Universty, New Haven, CT, [3]Biological Sciences, Universty of Tokyo, Tokyo, Japan, [4]Program in Molecular Medicine, University of Massachusetts, Worcester, MA, [5]Cell Biology, University of Massachusetts, Worcester, MA, [6]MMSB, University of Connecticut Health Center, Farmington, CT, [7]Cell Biology, Emory Universty, Atlanta, GA

The radial spoke is a ubiquitous component of “9+2” cilia and flagella and plays an essential role in the control of dynein arm activity by relaying signals from the central pair of microtubules to the arms. The *Chlamydomonas reinhardtii* radial spoke contains at least 23 proteins, only 8 of which have been characterized at the molecular level. Here we report the use of mass spectrometry to identify 10 additional radial spoke proteins. Many of the newly identified proteins in the spoke stalk contain domains associated with signal transduction, including calcium-, calmodulin-, nucleotide- and AKAP-binding domains. This suggests that the stalk is both a scaffold for signaling molecules and itself a transducer of signals. Moreover, in addition to the recently described HSP40 family member, a second stalk protein is predicted to be a molecular chaperone, implying

that there is a sophisticated mechanism for the assembly of this large complex. Among the 18 spoke proteins identified to date, 13 have apparent homologues in humans, indicating that the protein composition of the radial spoke has been conserved throughout evolution. The identification of human genes predicted to encode radial spoke proteins provides new candidates for causing primary ciliary dyskinesia, a severe inherited disease involving missing or defective axonemal structures, including the radial spokes.

1033

**The Parkin Co-Regulated Gene Product, PACRG, is an Evolutionarily Conserved Axonemal Protein that Functions in Outer Doublet Microtubule Morphogenesis**

H. Dawe, H. K. Farr, N. Portman, M. Shaw, K. Gull; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Eukaryotic cilia and flagella are highly conserved structures composed of a canonical 9+2 microtubule axoneme. Comparative genomics of flagellated and non-flagellated eukaryotes provides one way to identify new putative flagellar proteins. We analysed candidates emerging from such a screen by comparison with published human proteomic data and interrogated their putative flagellar function by RNA interference (RNAi) using the genetically tractable protozoan parasite *Trypanosoma brucei*. One such candidate was identified as the Parkin co-regulated gene product, PACRG. Male mice deficient in PACRG are sterile, but its function remains unclear. Bioinformatics revealed that *T. brucei* possesses two homologues of *PACRG*. We performed RNAi knockdown experiments of the two genes independently and together. Inducible RNAi knockdown of either protein individually had no effect on cell morphogenesis, growth or motility. However, simultaneous ablation of both proteins produced slow growth and paralysis of the flagellum with consequent effects on organelle segregation, suggesting functional redundancy. Transmission electron microscopy revealed structural defects in the axoneme, with microtubule doublets missing from the canonical 9+2 axoneme. The occurrence of missing doublets increased toward the distal end of the flagellum and sequential loss of doublets was observed along individual axonemes. Using videomicroscopy, we observed cells where the distal region of the flagellum was paralysed, although a beat was still observed in the proximal region of the flagellum. GFP fusion proteins of both PACRG homologues localised along the full length of the axoneme, but not to the basal body. Our results provide the first evidence for PACRG function within the axoneme, where we suggest that PACRG acts to maintain functional stability of the axonemal outer doublets of both motile and sensory cilia and flagella.

1034

**Characterization of the $SSTR_3$ Ciliary Targeting Signal**

Y. Vucica, G. Pazour; Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

The ciliary membrane is continuous with the apical plasma membrane but it contains a unique set of membrane proteins. Very little is known about ciliary targeting but a mechanism must exist that recognizes motifs in ciliary membrane proteins to target them for insertion into this membrane subdomain. To address this question, we have characterized the ciliary targeting motif in the $SSTR_3$ isoform of the somatostatin receptor. The $SSTR_3$ somatostatin receptor specifically localizes to primary cilia (Handel *et al*., Neurosci. 89:909-26). GFP-tagged $SSTR_3$ is highly localized to the ciliary membrane in cultured kidney cells, while the highly similar $SSTR_5$ isoform is not. Domain swapping experiments with $SSTR_5$ have identified the 19 amino acid extracellular loop between transmembrane helices 4 and 5 as the minimal sequence required for ciliary localization. This sequence is conserved in vertebrate $SSTR_3$ isoforms but not in other ciliary targeted membrane proteins. Thus, ciliary membrane targeting does not appear to be directed by common motifs as is observed for targeting to other organelles and to the basal-lateral surface of cells. Ciliary targeting appears more similar to apical membrane targeting where no common motif has been identified. In apical targeting, interaction with lipid rafts (driven by multiple mechanisms) appears to be the key step. Since the cilium projects from the apical surface of cells we are testing the idea that ciliary targeting is a specialized form of apical targeting. To do this, we are determining how the depletion of cholesterol, which disrupts lipid rafts, and how palmitoylation inhibitors, which can disrupt interaction of proteins with lipid rafts, affects the targeting of $SSTR_3$ to the cilium. Both perturbants affect ciliary targeting indicating that lipid raft association is likely to be a key event in ciliary targeting.

1035

**A Novel *Chlamydomonas* Mutant Having Flagellar Axonemes with Irregular Numbers of Outer Doublet Microtubules**

Y. Nakazawa, R. Kamiya, M. Hirono; Department of Biological Sciences, University of Tokyo, Tokyo, Japan

Almost all eukaryotic flagella and cilia have the same basic structure consisting of nine outer doublet microtubules and two central microtubules. How this structure is formed has been a long-standing question. We isolated a new *Chlamydomonas* mutant that has a defect in the basal body and assembles flagella with irregular numbers of doublet microtubules. This mutant, named *vdn1* for variable doublet number, grew slowly and displayed variable cell size. Approximately 89% of the cells were aflagellate, 9% uniflagellate, and 2% biflagellate. Electron microscopy revealed that, in most cells, basal bodies were split in pieces consisting of one to five triplets. However, all the axonemes possessed by this mutant consisted of doublet microtubules arranged in circles. Strikingly, the number of the doublets was variable between eight and eleven. Of the total of ~10,000 axonemal cross sections observed, 5% had eight doublets, 90% nine, 5% ten, and 2-3% eleven. All the eight-doublet axonemes contained no central pair microtubules. This is probably because an axoneme with such a small diameter with internally projecting radial spokes does not have space to accommodate central microtubules. In fact, the double mutant of *vdn1* with *pf14*, a mutant that lacks the radial spokes, displayed eight-doublet axonemes containing the central microtubules. In the axoneme with 10 or 11 doublets, the distance between the outer doublets and the central pair was not constant, resulting in a distorted arrangement of the doublets. In *vdn1 pf14*, however, no distortion was observed. From these observations, we concluded that the outer doublet microtubules in the axoneme have self-organizing properties to form a cylindrical structure, and that the radial spokes contribute to the assembly of the axonemal structure with a proper diameter.

1036

**HSP40 Is Required for the Final Assembly of Radial Spoke Complex in Flagella**

C. Yang,[1] D. R. Diener,[2] J. L. Rosenbaum,[2] H. A. Owen,[3] P. Yang[1]; [1]Department of Biological Sciences, Marquette University, Milwaukee, WI, [2]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, [3]Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI

Chaperone machinery has been implicated in the assembly of axonemes that occurs primarily at the tip of flagella. Recently, we have discovered

that a radial spoke protein, RSP16 in *Chlamydomonas* flagella is an HSP40. Interestingly, RSP16 is not a component of the 12S spoke precursor complex that is assembled in cell body and delivered to the flagellar tip by anterograde intraflagellar transport. Rather, in flagella RSP16 joins the precursors and becomes a constitutive component of the 20S stable spoke complexes. We postulate that RSP16 functions as a co-chaperone for converting precursors into mature spokes. To test this, we co-transformed wild type cells with one plasmid containing an RSP16-hairpin and the other conferring paromomycin-resistance. Among ~ 200 paromomycin-resistant transformants, four cells have flagella that jerk actively. Western analysis showed that RSP16 is not detected in their axonemes and cell body extract, while the other proteins in spokes, dyneins and central pair appear normal and the extracted radial spokes still sediment as 20S particles. Interestingly, negative stain electron microscopy reveals that the spokehead appears less compact. Consistently, chemical cross-linking of spokehead proteins is less efficient in RSP16-less spokes whereas the basal end of the spoke is not affected. Together, the results suggest that spoke HSP40 is specifically involved in the final folding of the head end of radial spokes; the improperly folded spokes in the absence of spoke co-chaperone results in defective interactions between spokes and central pair and, therefore, jerky flagellar beating.

## Cell Motility II (1037-1065)

1037

### Large-scale Spatial Coordination of Actin Meshwork Flow in Rapidly Moving Cells

C. A. Wilson,[1] P. T. Yam,[1] L. Ji,[2] G. Danuser,[2] J. A. Theriot[1]; [1]Biochemistry, Stanford University, Stanford, CA, [2]Cell Biology, The Scripps Research Institute, La Jolla, CA

The persistent movement of crawling cells depends on the proper spatial coordination of actin polymerization, actin depolymerization and myosin contraction, with actin polymerization biased toward the cell's leading edge and myosin contraction strongest at the rear. In the case of a motile cell, the entire cell coordinate system moves relative to the frame of reference in which the cell was observed. We use a rapid, non-iterative image cross-correlation approach to track cell rotation and translation, which is then used to define the relationship between cell and lab reference frames. This enables us to apply high-resolution tracking techniques including fluorescent speckle microscopy to measurements of the actin meshwork in rapidly moving cells such as fish keratocytes. We are characterizing, in both lab and cell reference frames, the coordination of F-actin flow dynamics across regions of cells undergoing different movement behaviors such as transient and sustained turns, and in the presence of drugs such as the myosin-II inhibitor blebbistatin. We find that cichlid keratocytes treated with blebbistatin do not lose polarity; however they do exhibit an accumulation of actin filaments at the rear and a slight decrease in speed. Blebbistatin also eliminates the inward (perpendicular to cell movement) flow of F-actin in the keratocyte rear and drastically reduces traction forces on deformable gelatin substrates. Thus, cell polarity and directional movement can be sustained solely by the actin polymerization-depolymerization cycle in keratocytes, with myosin playing a secondary role in generation of inward traction at the cell rear. Cell turning, however, appears to be strongly correlated with an imbalance in the relative rates of inward F-actin flow on the right and left sides at the rear of the cell, suggesting a strong role for myosin contraction in determining directional persistence.

1038

### Actin Rockets Guide Membrane Protrusion and Position Primed Integrins to Direct Pathfinding

C. G. Galbraith,[1] K. M. Yamada,[1] J. A. Galbraith[2]; [1]NIDCR, NIH, Bethesda, MD, [2]NINDS, NIH, Bethesda, MD

Migrating cells constantly probe their surrounding extracellular matrix in search of permissive adhesion sites where integrin adhesion receptors can connect the matrix to the force-generating actin cytoskeleton. The probing is not uniform along the advancing cell margin; adjacent regions of the leading edge are simultaneously advancing and retracting. However, the mechanism connecting the localized control of membrane protrusion with the function of probing for matrix ligand is unknown. Here we show that actin polymerizing as rockets along the leading edge guides local membrane protrusion and positions high affinity fibronectin receptors at discrete locations along the leading edge. The actin rockets move transversely along the leading edge, independent of retrograde actin network flow. The rockets are part of preferential actin polymerization in the direction of membrane protrusion, and their trajectories shape membrane protrusion in both fibroblast lamellae and neuronal growth cones. The transversely polymerizing actin also clusters beta1 integrins and drives synchronous integrin sideways movement, positioning conformationally activated but unligated beta1 integrins along the cell perimeter. The localization of activated beta1 integrins along the edge of fibroblast lamellae and at the tips of growth cone filopodia is dependent upon the availability of free actin ends. Our experiments establish that rapid transverse actin movement steers local changes in cell protrusions and positions primed integrin adhesion receptors at the leading edge, providing a mechanism for quickly sampling the permissiveness of the surrounding matrix to guide the cell toward new adhesion sites and preferred directions of migration.

1039

### Plasma Membrane Organization is Essential for Balancing Competing Pseudopod- and Uropod- Promoting Signals During Neutrophil Polarization and Migration

S. F. Bodin, M. D. Welch; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Exposure of neutrophils to chemoattractant induces cell polarization and migration. These behaviors require the asymmetric activation of distinct signaling pathways and cytoskeletal elements in the protruding pseudopod at the front of cells, and the retracting uropod at the rear. An important outstanding question is, how does the organization of the plasma membrane participate in establishing and maintaining asymmetry during polarization and migration? To answer this question, we investigated the function of cholesterol, a lipid that is known to influence the organizational properties of the membrane. Using controlled cholesterol depletion to alter membrane composition, we found that a cholesterol-dependent membrane organization played an essential role in cell polarization and migration by enabling uropod function and suppressing ectopic pseudopod formation. At the molecular level, cholesterol-dependent membrane organization was required for the activation of RhoA, a major determinant of uropod activity. Moreover, it was essential for restraining to a single membrane area the accumulation of D3-phosphoinositide lipids, which serve as critical cues for pseudopod formation. At a mechanistic level, cholesterol was directly required to suppress the activation of $G_i$, and in turn to suppress inappropriate PI3-kinase activation while permitting the activation of RhoA. Our findings suggest a model in which a cholesterol-dependent membrane organization plays an essential role in the establishment of cellular asymmetry by balancing the activation and

segregating the localization of competing pseudopod- and uropod-inducing signaling pathways during neutrophil polarization and migration.

1040

**A Specific Role of Integrin Mac-1 in Inflammatory Macrophage Migration**

C. Cao, Y. Li, L. Zhang; Physiology, University of Maryland School of Medicine, Baltimore, MD

A Specific Role of Integrin Mac-1 in Inflammatory Macrophage Migration In response to acute inflammation, monocytes/macrophages accumulate at the site of injury, where they participate in various biological processes. The number of inflammatory monocytes/macrophages within the injury site increases during the early phase of inflammation and then returns to basal level when inflammation is resolved. However, the mechanism underlying the disappearance of these inflammatory monocytes/macrophages is not fully understood. In this study we examined the accelerated migration of inflammatory macrophages from the inflamed peritoneum to the draining lymph nodes, using a thioglycollate (TG)-induced peritonitis model for in vivo and a Boyden-chamber type transwell plate for in vitro migration assays. We found that unlike neutrophils the inflammatory macrophages did not undergo apoptosis within the site of inflammation. Instead, they migrated across the peritoneal mesothelium into the lymphatics at the end of acute inflammation, and from there they further moved to the lymph nodes and to the blood circulation. Most importantly, we found that genetic inactivation of Mac-1 in mice abolished this accelerated macrophage efflux from the inflammatory site to the lymphatics, but not the accumulation of blood monocytes into the inflamed peritoneum. In summary, our study demonstrates that Mac-1 is involved specifically in the efflux of activated inflammatory macrophages to the lymphatics, suggesting that Mac-1 may play an important role in the removal of local inflammatory macrophages and in their subsequent migration to the lymph nodes, a process that is critical to the development of the adaptive immunity

1041

**mDia1 Regulates Cell Adhesion and Microtubule Stabilization During the Migration of Chick Embryo Cardiac Fibroblasts**

L. H. Tahtamouni,[1,2] L. P. Cramer,[3] J. R. Bamburg[1]; [1]Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, [2]Biology, The Hashemite University, Amman, Jordan, [3]MRC-Laboratory for Molecular Cell Biology and Department of Biology, University College London, London, United Kingdom

Establishing polarized migration involves spatial and temporal reorganization of actin and microtubules. mDia1, a member of the formin protein family, coordinates both actin and microtubule cytoskeletons through its FH1-FH2 unit. We examined effects of both constitutively active (CA) and dominant negative (DN)-mDia1 on polarized migration of chick embryo cardiac fibroblasts (CCFs). Twenty-four hours prior to plating, cardiac explants in suspension were infected with recombinant AdTrack adenovirus to express the protein of interest. Polarized cells comprised 52% of uninfected and control infected cells, but only 24.9% of CAmDia- and 4.8% of DNmDia-expressing cells. Directional migration in control CCFs was maintained for a minimum of 15 min at an average rate of 1.14 mm/min, whereas polar and bipolar cells expressing CA-mDia1 migrated at 0.49 μm/min and 0.11 μm/min, respectively. Overexpression of CAmDia1 did not alter the G-actin/F-actin ratio in CCFs, but it increased the size, number, and total area of focal adhesions in CCFs. It also caused a 71% increase in the number of microtubule plus ends/unit area penetrating the lamellipodium, 65% of which were stabilized. Most cells expressing DN-mDia1 failed to migrate out of the explants and DNmDia expression in dissociated cells prevented their attachment. Co-expression of paxillin restored cell adhesion but not polarized migration. Together, our data demonstrate that mDia1 regulates cell adhesion and microtubule dynamics in CCFs. (Supported by NIH grants GM35126 and NS40371 (JRB), a Royal Society Fellowship and Wellcome Trust grant (LC), and a Jordanian government/The Hashemite University fellowship (LT)).

1042

**α4 Integrin Phosphorylation as a Mechanism to Regulate Cell Migration**

L. A. Rivera Rosado, J. T. Yang; Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

α4β1 integrin confers motility to many cell types *in vivo* and to cells in culture. In the present study we proposed that phosphorylation at the cytoplasmic tail of the α4 integrin subunit (α4 tail) may directly activate an intracellular signaling pathway to regulate cell migration. We and others have shown that, in response to scratch-wounding, the Y991A mutation, which reduces paxillin binding to α4, enhances the lamellipodia-promoting activity of α4β1, whereas the S988A mutation, which abolishes $Ser^{988}$ phosphorylation in the α4 tail, impairs this activity. To determine whether α4 phosphorylation regulates paxillin-dissociation, which in turn promotes migration, or directly activates downstream signals, an epistasis test was performed using CHO cells stably expressing α4 carrying both Y991A and S988A mutations. The double mutant (DM) α4 tail was not recognized by the phospho-α4 antibody and did not co-immunoprecipitated paxillin, indicating that both α4 phosphorylation and paxillin binding were disrupted. To determine the effect of α4-DM on the lamellipodia-promoting activity of α4β1, CHOα4-DM cells were tested in the scratch-wound assay. We found that CHOα4-DM resulted in the S988A phenotype, impairing the ability of α4 to promote lamellipodia protrusion, and closed scratch-wounds slower than CHOα4-GFP and CHOα4Y991A-GFP cells. These results indicate that paxillin dissociation from the α4 tail is not sufficient to promote cell migration and that phosphorylation at $Ser^{988}$ is required for the lamellipodia-promoting activity of α4β1 even in the absence of paxillin binding. These data support the hypothesis that α4 phosphorylation plays an active role in activating downstream signaling and promoting lamellipodia protrusion.

1043

**A Wnt-Like Pathway Controlling Polarity during Cell Migration**

K. Schlessinger,[1] J. Cau,[1] E. J. McManus,[2] D. R. Alessi,[2] A. Hall[1]; [1]MRC-LMCB, University College London, London, United Kingdom, [2]MRC Protein Phosphorylation Unit, University of Dundee, Dundee, United Kingdom

Wound-induced directed cell migration leads to reorientation of the Golgi and the MTOC. The signaling events controlling this process require localized activation of Cdc42 and the Par6/PKCζ complex at the leading edge. GSK3β is coordinately phosphorylated on Ser9, leading to APC release from GSK3β and binding to microtubule plus ends, thereby stabilizing microtubules in the direction of migration. GSK3 can be inactivated through Ser phosphorylation, which occurs during insulin signaling, or by protein-protein interaction, which occurs during Wnt signaling and requires Dishevelled (Dvl). To investigate the role of GSK3 phosphorylation in reorientation, GSK3α/β Ser to Ala double knockin fibroblasts (GSK3-KI) were used. Surprisingly, GSK3-KI cells had no defect in Golgi/MTOC reorientation. However, GSK3 inhibitors did block reorientation

in GSK3-KI cells, suggesting that GSK3 is required for reorientation in a phosphorylation-independent manner. To test whether GSK3 inactivation occurs through Dvl interaction, Dvl2 was microinjected into migrating fibroblasts. Reorientation was blocked in cells over-expressing Dvl2, suggesting that Dvl may be involved. When different Dvl mutants were injected, only constructs containing the DIX domain were able to block reorientation, implying that Dvl interaction with Axin or another Dvl molecule are required for reorientation. Coimmunoprecipitation experiments confirmed a complex formation between different Dvl isoforms following wounding, and Dvl depletion using siRNA also led to defects in reorientation. Our data suggest that Dvl is required for Golgi/MTOC reorientation, and therefore GSK3 inactivation through Dvl, rather than phosphorylation, may be the mechanism regulating reorientation during directed cell migration. The similarity to Wnt signaling prompted further investigation into the possible involvement of Wnt ligands and Frizzled receptors in this process.

1044

**PDZ-dependent Binding is Essential for Rho Guanine Exchange Factor Function and Cell Migration**

M. Liu, A. Horowitz; Angiogenesis Research Center, Dartmouth Medical School, Lebanon, NH

We have previously reported that a recently characterized RhoA-specific guanine exchange factor (GEF) was expressed as two splice variants, where the C-terminus of one variant (SYnectin-interacting eXchange factor 1 - Syx1) conformed to the consensus sequence of class 1 PDZ motifs and bound the PDZ adaptor protein synectin, while the other (Syx2) did not, as it was shorter by two C-terminal amino acids (MBC 14:285a, 2003). Overexpression of each splice variant in endothelial cells (EC) produced contrasting effects: EC overexpressing Syx1 migrated significantly faster than EC overexpressing Syx2. Silencing of the expression of endogenous Syx1, which is the predominant Syx isoform in EC, reduced global RhoA activity by ~50%, indicating that Syx1 is a major RhoA agonist in EC. Since Syx1 binds synectin, we examined the dependence of EC migration on the expression level of the latter protein. Silencing endogenous synectin expression in EC that overexpressed Syx1 reduced their migration by 80%. Similar to endogenous Syx, YFP-Syx1 was localized in the perinuclear region and at the plasma membrane (PM), where it collocated with synectin. YFP-Syx2, however, was diffusely distributed in the cytoplasm. In EC from synectin-null cells, endogenous Syx1 assumed a diffuse distribution pattern similar to that of overexpressed YFP-Syx2, indicating that Syx1 targeting is dependent on binding to synectin. Though global RhoA activity levels in EC overexpressing Syx1 or Syx2 were similar, their spatial activity patterns were drastically different. FRET experiments revealed that active RhoA in Syx1-overexpressing EC was located at the PM, but in Syx2-overexpressing EC it was diffusely distributed in the cytoplasm, similar to the localization of Syx2. These results show that the interaction of the C-terminus PDZ-binding motif of Syx1 with synectin is essential for its targeting and its promotion of cell migration.

1045

**PTP-PEST Mediates Cell Spreading and Motility through an Interaction with Paxillin**

J. S. Jamieson,[1] D. A. Tumbarello,[1] M. Hallé,[2] M. C. Brown,[1] M. L. Tremblay,[2] C. E. Turner[1]; [1]Dept. of Cell & Developmental Biology, SUNY Upstate Medical University, Syracuse, NY, [2]McGill Cancer Centre, McGill University, Montreal, PQ, Canada

The tyrosine phosphatase PTP-PEST has been implicated in the regulation of cell spreading and migration through dephosphorylation of focal adhesion proteins and inhibition of Rac GTPase activity (Sastry et al, JCS, 2002, 115:4305-16). Paxillin, a focal adhesion adaptor protein, is also required for cell spreading and motility (Brown and Turner, Physiol Rev, 2004, 84:1315-39). In this study we use paxillin -/- and PTP-PEST -/- fibroblasts to show that the paxillin-PTP-PEST interaction, via the LIM 3 and 4 domains of paxillin and the Pro 2 domain of PTP-PEST, is required for PTP-PEST to inhibit spreading and protrusion. In addition, we show that paxillin is required for stimulation of migration in reconstituted PTP-PEST -/- fibroblasts. Furthermore, using PBD analysis, we demonstrate that paxillin is essential for PTP-PEST to inhibit adhesion-induced Rac1 activity. Mutational analysis of paxillin indicates that PTP-PEST regulation of spreading and protrusion involves an interaction with the LIM domains in the carboxyl-terminus signaling via two regions in the amino-terminus: the LD4 motif and the tyrosine phosphorylation sites 31 and 118. These domains couple paxillin to the regulation of Rac1 activity via interaction with PKL-PIX-PAK and Crk respectively (Brown and Turner, Physiol Rev, 2004, 84:1315-39). Using “substrate trapping” approaches and immunoprecipitation analysis we show PKL to be a potential target of PTP-PEST. Additionally, we show that a PKL-paxillin interaction is required for PTP-PEST to regulate spreading. Together these results provide insight into the role of the paxillin-PTP-PEST interaction in integrin signaling events. Supported by NIH GM47606.

1046

**PKA Regulates Abl Function During Chemotaxis**

P. B. Deming, L. C. Baldor, A. K. Howe; Pharmacology, University of Vermont, Burlington, VT

The cAMP-dependent protein kinase (PKA) plays important roles in cytoskeletal re-organization and cell migration. Spatial regulation of PKA is an critical component of chemotactic cell movement as disruption of either PKA activity or anchoring impairs pseudopod formation. In response to growth factor, PKA phosphorylates the vasodilator stimulated phosphoprotein (VASP) within pseudopodia which disrupts its interaction with the Abl tyrosine kinase. Abl is also an important modulator of cytoskeletal dynamics and is thought to exert a negative effect on cell migration. This suggests that PKA may exert its effects on cell migration, at least in part, through spatial regulation of Abl function. Intriguingly, we have observed that like PKA, Abl protein is also spatially regulated during chemotaxis. While Abl protein was enriched in pseudopods, the kinase activity of Abl - as assessed by phosphorylation of tyrosine 221 of the Crk adaptor protein - was not increased in pseudopodia. Inhibition of PKA activity in pseudopods resulted in an increased phosphorylation of endogenous Crk on Tyr221. Moreover, biochemical fractionation analyses imply that PKA may modulate the subcellular localization of Abl. Given our recent demonstration that PKA activity presides over Rac activation in pseudopods and the central role of Rac in chemotactic cell migration, our current data suggest that PKA may regulate Rac activity via modulating Abl function during chemotactic cell movement.

1047

**Inhibition of Serine/Threonine Kinases Stimulates Proliferation and Migration in Polyamine Depleted Intestinal Epithelial Cells**

H. Guo, M. Patel, R. M. Ray, L. R. Johnson; Department of Physiology, University of Tennessee Health Science Center, Memphis, TN

Previously, we showed that polyamine-depletion inhibits proliferation and migration in intestinal epithelial cells (IEC-6). We also found that polyamine depletion inhibits protein phosphatase 2A (PP2A) activity. From these results, we hypothesized that polyamine-depletion inhibits

proliferation and migration by increasing serine/threonine phosphorylation. Staurosporine (STS) is a potent, cell permeable, and broad-spectrum serine/threonine kinase inhibitor. We determined whether STS-induced inhibition of serine/threonine kinases in polyamine-depleted cells restores proliferation and migration. Cells grown in the presence of DFMO (polyamine depletion) showed a significantly higher proportion of multi-nucleated cells indicating defective cytokinesis. In polyamine depleted cells, p-MLC (phospho myosin light chain) (Thr 18/Ser19) localized with a thick actin cortex. Although, 5 nM STS almost completely restored migration in polyamine depleted cells within 4h, prolonged exposure showed apoptotic effects. However, 1 nM STS restored migration within 7 h without any sign of apoptosis. Polyamine depletion inhibited lamellipodia formation, cytokinesis, and sequestered the p- MLC with the actin cortex. STS caused dissolution of the actin cortex, leading to the formation of lamellipodia in polyamine-depleted cells. STS significantly inhibited phosphorylation and the subsequent localization of MLC at the cell periphery. STS treatment increased Rac1 activity in polyamine-depleted cells. Cells grown in the presence of DFMO plus 0.1nM STS had a significantly higher rate of proliferation compared to cells grown in the presence of DFMO. These observations indicate that balance between serine/threonine kinases and serine/threonine phosphatases play a critical role in the regulation of proliferation and migration, and that it is altered in polyamine depleted cells.

1048

### Central Role of the Phosphatidylinositol-3-kinase Signalling Pathway in the Regulation of Human Gastric Epithelial Cell Migration

M. Tétreault, P. Chailler, N. Rivard, D. Ménard; Anatomy and Cell Biology, Université de Sherbrooke, Sherbrooke, PQ, Canada

The integrity of the human gastric mucosa is maintained through its highly-dynamic regenerative capacity upon wounding. Our previous studies established that growth factors (GFs : TGFα, HGF) differently regulate the regeneration of human gastric epithelial cells. However, the intracellular signaling pathways that transmit extracellular cues and that regulate basic and stimulated gastric epithelial cell migration are still unclear. **Aim**: To investigate the involvement of PI3K signalling pathway in the regulation of gastric epithelial migration. **Methods**: Confluent HGE-17 cell monolayers were wounded with a razor blade and migration was quantified by counting the number of cells across the wound margin. The effects of a synthetic form of PtdIns(3,4,5)$P_3$ and of pharmacologic inhibitors (LY294002, rapamycin, toxinB) were examinated with and without growth factors. Wounded cultures were lyzed at different time intervals and the activation profiles of Akt and p70S6K were analyzed. The biological consequences of wild-type Akt (wt-Akt) and myristylated Akt (myr-Akt) overexpression in HGE-17 cells were studied. **Results:** LY294002 (PI3K inhibitor) strongly decreased basal and GFs-induced cell migration (40-70%) and this inhibition was already significant after only 60 min exposure. In accordance, treatments with PtdIns(3,4,5)$P_3$ greatly accelerated cell migration by 230%. Putative targets such as Akt and p70S6K were all stimulated in a PI3K-depedent manner by wounding and GFs. Specific inhibitors of RhoGTPase and mTOR/p70S6K significantly decreased HGE-17 cell migration by 40% and 25% respectively. Even though wounding induced Akt activation as early as 5 min, overexpression of wt-Akt and myr-Akt did not accelerated migration. **Conclusion :** Activation of PI3K is a central and early event involved in the control of basal and GFs stimulated gastric cell migration but the intracellular targets responsible for the differential actions of GFs are probably downstream in the pathway.

1049

### JSAP1/JIP3 Cooperates with FAK to Regulate C-jun N-terminal Kinase and Cell Migration

T. Takino,[1] H. Miyamori,[1] K. M. Yamada,[2] H. Sato[1]; [1]Molecular Virology and Oncology, Cancer Research Institute, Kanazawa, Japan, [2]CDBRB, NIDCR/NIH, Bethesda, MD

c-Jun N-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1) (also termed JNK interacting protein 3; JIP3) is a member of a family of scaffold factors for the mitogen-activated protein kinase (MAPK) cascades, and it also forms a complex with focal adhesion kinase (FAK). Here we demonstrate that JSAP1 serves as a cooperative scaffold for activation of JNK and regulation of cell migration in response to fibronectin (FN) stimulation. JSAP1 mediated an association between FAK and JNK, which was induced by either co-expression of Src or attachment of cells to FN. Complex formation of FAK with JSAP1 and p130 Crk-associated substrate ($p130^{Cas}$) resulted in augmentation of FAK activity and phosphorylation of both JSAP1 and $p130^{Cas}$, which required $p130^{Cas}$ hyperphosphorylation and was abolished by inhibition of Src. JNK activation by FN was enhanced by JSAP1, which was suppressed by disrupting the FAK/$p130^{Cas}$ pathway by expression of a dominant-negative form of $p130^{Cas}$ or by inhibiting Src. We also documented the co-localization of JSAP1 with JNK and phosphorylated FAK at the leading edge and stimulation of cell migration by JSAP1 expression, which depended on its JNK binding domain and was suppressed by inhibition of JNK. The level of JSAP1 mRNA correlated with advanced malignancy in brain tumors, unlike other JIPs. We propose that the JSAP1/FAK complex functions cooperatively as a scaffold for the JNK signaling pathway and regulator of cell migration on FN, and we suggest that JSAP1 is also associated with malignancy in brain tumors.

1050

### The Role of Caveolae Endocytosis in Caveolin-1 Polarization in Migrating Endothelial Cells

V. Santilman,[1] J. Baran,[1] M. A. McNiven,[2] M. Parat[1]; [1]Center for Anesthesiology Research, Cleveland Clinic Foundation, Cleveland, OH, [2]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN

Endothelial cell migration is a critical event in angiogenesis. Caveolae as well as their major protein caveolin-1 are polarized in migrating cells and the polarization of caveolin-1 varies with the migration mode (planar migration versus transmigration). Tyr-14 residue of the protein is necessary for this polarization. The phosphorylation of Tyr-14 of caveolin-1 is thought to be involved in a number of processes such as caveolin-1 -protein and -lipid binding and caveolae endocytosis. In this work, we wanted to test the requirement of caveolae endocytosis for caveolin-1 polarization in migrating endothelial cells. For this purpose, we inhibited the internalization of caveolae using a dominant negative mutant of dynamin-2 (K44A mutant). This mutant lacks normal GTPase activity and its expression inhibits GTP-induced fission, budding and internalization of caveolae. We verified its efficiency to inhibit caveolae endocytosis in bovine aortic endothelial cells (BAEC) by looking at bovine serum albumin and cholera toxin internalization. We examined the localization of caveolin-1 in dynamin-transfected cells migrating in two or three dimensions. For the transmigration assay, BAEC were placed in the top well of a Boyden Chamber and allowed to migrate across the pores of a collagen-coated polycarbonate filter. The localization of caveolin-1 was detected by immunofluorescence in transfected cells identified by the expression of WT or mutant dynamin-2-GFP. The K44A dynamin-2 mutant effectively inhibited caveolae endocytosis in BAEC. Our data indicate that the inhibition of caveolae internalization does not decrease the rate of planar migration or transmigration but, on the contrary, increases the rate of transmigration.

Moreover, the expression of the mutant dynamin-2 does not disturb the localization of caveolin-1 in either migration mode. Our results indicate that caveolae endocytosis is not critical for the polarization of caveolin-1 in migrating endothelial cells.

1051

**Collective Migration of Epithelial Cells**

R. Farooqui, G. Fenteany; Chemistry, University of Illinois at Chicago, Chicago, IL

The mechanism by which epithelial, endothelial and other strongly cell-cell adhesive cells migrate collectively as continuous sheets is not clear, even though this process is critical for embryonic development and tissue repair in virtually all multicellular animals. We used wound closure in Madin-Darby canine kidney (MDCK) epithelial cell monolayers as a model to study migration of cells both at and behind the wound edge. We report here for the first time that cells behind the margin of wounded MDCK cell monolayers, even hundreds of microns from the edge, extend "cryptic" lamellipodia against the substratum beneath cells in front of them toward the wound, as determined by confocal, two-photon and transmission electron microscopy. These so-called submarginal cells nevertheless strictly maintain their more apical cell-cell contacts as they migrate as part of a coherent cell sheet, hiding their basal protrusions from conventional microscopy. The submarginal protrusions display the hallmarks of traditional lamellipodia based on morphology and dynamics. The rate of migration is inversely proportional to distance from the margin, and cells move coordinately yet still partly autonomously toward the wound area. We also clarify the ancillary role played by non-protrusive contractile actin bundles that assemble in a Rho GTPase-dependent manner at the margin after wounding. In addition, some cell proliferation occurs at a delay after wounding but does not contribute to closure, instead apparently serving to replace damaged cells so that intact spread cells can revert to their normal cuboidal morphology and the original cell density of the unbroken sheet can be restored. Cells behind the margin therefore actively crawl, instead of just moving passively as cells at the margin pull on them.

1052

**Coupling Actin Cytoskeleton Dynamics to Substrate Adhesion in Migrating Epithelial Cells**

S. L. Gupton, C. M. Waterman-Storer; Cell Biology, Scripps Research Institute, La Jolla, CA

Cell migration is mediated by the formation of ECM ligand - receptor complexes in the cell front and their weakening and disassembly in the cell rear. The receptors assemble with structural and signaling proteins to form focal adhesions, complexes that link the intracellular actin cytoskeleton to the ECM, allowing myosin-driven tension in the cytoskeleton to produce traction against the ECM and drive cell movement. Cells show biphasic migration velocity in response to increasing adhesion strength, indicating an optimal adhesion strength exists for migration. In this study we sought to determine if adhesion-dependent changes in migration velocity are mediated by distinct dynamic states of focal adhesions and the actin cytoskeleton. We show changes in the lamellipodium module do not correlate with cell migration, but rather do changes in the lamella contractile module of the cell. We find evidence for a "clutch" linking lamella actin retrograde flow to the substrate, and suggest tuning this clutch modulates actin flow and migration. We characterize an optimal actin phenotype for migration, which includes rapid actin convergence coupled to rapid actin depolymerization in the convergence zone, which is likely driven by increased bound and active myosin. We show rapid renewal of focal adhesions components coupled to intermediate focal adhesion lifetime and turnover rates correlate with fast cell migration. These studies indicate that simple models for modulation of cell biphasic migration velocity are mediated by a complex spatio-temporal feedback between adhesion sites and myosin activity driving the organization of an actin cytoskeleton optimized for directed cell migration.

1053

**The Shwachman-Diamond Syndrome Gene Encodes an RNA-Binding Protein That Localizes to the Pseudopod of Dictyostelium Amoebae During Chemotaxis**

D. Wessels,[1,2] T. Srikantha,[1] S. Yi,[1] S. Kuhl,[1] L. Aravind,[3] D. R. Soll[1]; [1]Dept. Biological Sciences, University of Iowa, Iowa City, IA, [2]Dept. Biological Sciences, W.M.Keck Dynamic Image Analysis Facility, Iowa City, IA, [3]NIH, Computational Biology Branch NCBI, Bethesda, MD

The Shwachman-Diamond Syndrome (SDS) is an autosomal disorder with multisystem defects. The gene *SBDS*, which contains mutations in a majority of SDS patients, encodes a protein with a deduced function in RNA metabolism. Recently it was demonstrated by computer-assisted methods that the single behavioral defect of polymorphonuclear leukocytes (PMNs) of SDS patients is the incapacity to orient correctly in a spatial gradient of chemoattractant. Although the deduced function of the *SBDS* gene product is in RNA metabolism, the very specific behavioral defect exhibited by PMNs of SDS patients suggested that it played a role in chemotactic orientation. Given that several proteins involved in chemotactic orientation localize to the pseudopod of cells during chemotaxis, we tested whether the *SBDS* gene product did the same. We identified the homolog of *SBDS* in the social amoeba *Dictyostelium discoideum*, a model for PMN chemotaxis, produced an *SBDS*-GFP chimeric in-frame fusion gene, and generated transformants either with multiple ectopic insertions of the fusion gene or multiple copies of a non-integrated plasmid carrying the fusion gene. The *SBDS*-GFP protein was dispersed equally through the cytoplasm and pseudopods of cells migrating in buffer, but exhibited differential localization in the pseudopods of cells undergoing chemotaxis in a spatial gradient of chemoattractant in both types of transformants, suggesting that the SBDS protein may play a role in chemotactic orientation. Pseudopod localization was demonstrated to be a result simply of chemotactic receptor occupancy, not a result of chemotaxis per se. To our knowledge, SBDS represents the first protein involved in RNA metabolism that localizes to pseudopods in response to a chemoattractant.

1054

**Two Putative WAVE Associated RacGAP (WRP) Proteins Are Important in All Aspects of *Dictyostelium discoideum* Motility**

R. J. W. Heath, R. H. Insall; School of Biosciences, The University of Birmingham, Birmingham,, United Kingdom

Cellular movement and shape change is dependent upon cycles of actin polymerization and depolymerization. The Arp2/3 complex is a principal component of this process, nucleating actin filaments at the side of existing filaments. WRP (Soderling *et al*, 2002) has recently been identified in mammalian tissues as a binding partner of SCAR/WAVE1 and is a proposed signal termination factor for Rac but has no affect on Rho or CDC42. Here we identify two putative *D. discoideum* WRP proteins. We have disrupted both WRP1 and WRP2 genes by homologous recombination and made a WRP1$^-$/2$^-$ double knockout. We find a strong cytoskeletal phenotype in WRP1$^-$ cells and a relatively subtle chemotaxis defect in WRP2$^-$ cells. WRP1$^-$/2$^-$ double mutant cells have a seriously aberrant cytoskeleton and drastically impaired motility. In addition WRP1$^-$ slugs have a subtle

phototaxis phenotype while WRP2$^-$ slugs are totally defective in phototaxis and WRP1$^-$/2$^-$ slugs fail to migrate at all. The development of all WRP$^-$ cell lines is also affected. WRP2$^-$ cells develop slightly earlier than wild type cells, WRP1$^-$ fruiting bodies have short, thin stalks and the WRP1$^-$/2$^-$ double mutant line fails to form differentiated fruiting bodies. WRP is clearly important in linking external signals to the cytoskeleton; however, we find little evidence for a genetic interaction with SCAR.

1055

**Distinct Mechanisms Regulate Hemocyte Chemotaxis During Wound Healing and Development in Drosophila**

W. Wood, C. Faria, A. Jacinto; Instituto de Medicina Molecular, Lisbon, Portugal

*Drosophila* hemocytes are highly motile macrophage-like cells that undergo a stereotypic pattern of migration to populate the whole embryo by late embryogenesis. Our results demonstrate that the migratory patterns of hemocytes at the embryonic ventral midline are orchestrated by chemotactic signals from Pvf signals. Also, these directed migrations seem to be independent of Phosphoinositide 3-Kinase (PI3K) signalling as shown by the overall distribution of hemocytes expressing a PI3K Dominant Negative form and by a detailed quantification of both cell directionality and velocity. In contrast we show, using both laser ablation and a novel wounding assay that allows localised treatment with inhibitory drugs, that PI3K is essential for hemocyte chemotaxis towards wounds and that Pvf signalling is not required for this rapid chemotactic response. Our results imply that at least two separate mechanisms operate in *Drosophila* embryos to direct hemocyte migration, and whilst PI3K is crucial for hemocytes to sense a chemotactic gradient from a wound it is not required to sense the signals that coordinate their developmental migrations along the ventral midline during embryogenesis.

1056

**Glutamate in Neutrophil Activation: A New Role in Wound Healing Process**

R. Gupta,[1,2] D. Chattopadhyay[1,2]; [1]Department of Biochemistry, University of Calcutta, Calcutta, India, [2]Department of Biochemistry, Dr. B. C. Guha Center for Genetic Engineering and Biotechnology, Calcutta, India

Human placental extract has been found to be efficacious against wound healing for a long time. When a wound is inflicted, neutrophils migrate to the wound site within minutes of injury; to clear up the infection by engulfing the bacteria and also as the source of pro-inflammatory cytokines, which activate the surrounding fibroblasts and keratinocytes. HPLC analysis of the hot aqueuos human placental extract revealed Glutamate to be the predominant free amino acid. We investigated the role of glutamate as a chemoattractant with particular attention to its ability to activate neutrophil responses. Boyden chamber assay and FACS (F-actin quantification with phalloidin) analysis revealed the induction of chemotaxis and actin polymerization respectively optimally at a concentration of 8 µM of glutamate. A time-lapse videomicroscopy showed the polarization of neutrophils with the addition of glutamate in a span of 500 seconds. Immunofluorescence with phalloidin staining revealed the polarization of actin cytoskeleton with glutamate addition. The glutamate induced chemotaxis and the related events were found to be PI3K dependent phenomena as phospho-TLC showed the induction of PIP3 and the PI3K inhibitors wortmannin, LY29004 abrogated the glutamate effect. The translocation of GFP fluorescence to plasma membrane by a global glutamate stimulation following a PH-Akt-GFP construct electroporation reconfirmed the PI3K dependent pathway. Rac2 activation was also observed with 8µM glutamate. Incubation of neutrophils with glutamate (8 µM-32 µM) triggered oxidative burst after plating on fibrinogen. At the receptor level, the glutamate effect was actually a pertussis toxin insensitive effect, with a non-involvement of the characteristic chemoattractant G-protein-coupled receptors. Blocking antibodies to integrin beta 2 totally abrogated the glutamate signaling. Taken together, these results suggest a role for the glutamate as a chemoattractant, which is able to induce neutrophil activation.

1057

**Formin-binding Protein 2 (FNBP2) as a Candidate Regulator of Neuronal Migration in the Cortex**

S. Guerrier, F. Polleux; Department of Pharmacology, University of North Carolina-Chapel Hill, Chapel Hill, NC

The proper formation of functional neuronal circuits within the central nervous system is critically dependent upon the coordinated migration of two types of neurons, glutamatergic pyramidal neurons (excitatory), and GABAergic interneurons (inhibitory). Defects in the migration of these neurons play a role in several neuropathologies including schizophrenia and epilepsy. Formin Binding Protein 2 (FNBP2) was recently identified as a transcriptional target of Neurogenin 2, a basic-helix-loop-helix transcription factor that we have shown to regulate the migration properties of glutamatergic neurons in the developing cortex. FNBP2 is a member of the Slit/Robo GTPase activating protein family and was initially identified through its interaction with the Robo receptor. In situ hybridization indicates that FNBP2 is expressed in the ventricular zone in the developing cortex, where glutamatergic neurons initiate radial migration. Protein motif analysis indicates that FNBP2 is composed of 3 domains, an Fes/Cip4 homology (FCH) domain, rho GTPase activating (GAP) domain and a Src homology 3 (SH3) domain. These domains have been previously characterized as regulators of the cytoskeleton and protein-protein interactions, however the role of these domains in mediating the function of FNBP2 has not been determined. In this study, we aimed to determine the role of FNBP2 in the migration of cortical glutamatergic neurons. Our preliminary results demonstrate that overexpression of FNBP2 causes significant cytoskeletal rearrangement in cos7 cells. Furthermore, overexpression of FNBP2 in cortical neurons using electroporation-mediated gene transfer impairs their migration in a slice culture assay. These results suggest that FNBP2 might regulate cortical migration by controlling cytoskeletal dynamics. Current investigation involving structure-function analysis as well as in vivo loss-of-function analysis will allow us to determine the mechanism underlying FNBP2 regulation of cytoskeletal dynamics during neuronal migration

1058

**Disruption of Centrosome Positioning Ahead of Nucleus Impairs Leading Process Outgrowth and Nuclear Translocation during Neuronal Migration**

H. R. Higginbotham,[1] T. Tanaka,[2] B. C. Brinkman,[2] J. G. Gleeson[2]; [1]Biomedical Sciences, University of California, San Diego, La Jolla, CA, [2]Dept. of Neuroscience, University of California, San Diego, La Jolla, CA

In comparison with other migratory cells, neurons exhibit a unique, highly polarized morphology and a distinctive pattern of movement. The leading process extends forward in the direction of migration and subsequently the nucleus translocates into the leading process, but the events

controlling these phases are not known. In olfactory bulb neuronal precursors we find that nuclear translocation occurs in two distinct stages: first, the centrosome moves into the leading process, which coincides with process extension, then the nucleus follows, moving abruptly towards the centrosome. Application of the chemorepellent Slit, which reverses cell polarity, first leads to establishment of a new process, then reorientation of the centrosome followed by nuclear translocation in the reverse direction. Inhibition of either GSK-3b or PKC-zeta, critical cell polarity factors, resulted in impaired process outgrowth and centrosome reorientation, with resultant failure in nuclear translocation in the reverse direction. Our findings suggest that positioning of the centrosome ahead of the nucleus is an important step in directed neuronal migration and repolarization in response to guidance cues.

1059

**Role of Glycogen Synthase Kinase - 3 in Sperm Function Examined with Lithium Chloride**

P. Puri,[1] S. P. R. Shenoy,[2] D. W. Kline,[1] S. Vijayaraghavan[1]; [1]Department of Biological Sciences, Kent State University, Kent, OH, [2]Department of Molecular Cardiology,, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

Testicular spermatozoa are immotile and cannot bind to the egg. Sperm undergo maturation during their passage through the epididymis attaining motility and fertility potential. Biochemical mechanisms underlying sperm maturation are not completely understood. We suggested that signaling mechanisms involving serine threonine phosphatases and glycogen synthase kinase-3 may be involved in motility initiation. Both α and β isoforms of GSK-3 are serine phosphorylated in sperm. The upstream regulators of GSK-3, PI-3 kinase and Akt are also present in spermatozoa. We have examined the role of GSK-3 in sperm motility using the GSK-3 inhibitor, lithium chloride (LiCl). Motility was measured using computer assisted semen analysis. Lithium chloride dramatically decreased all motility parameters of bovine caudal epididymal spermatozoa in a concentration dependent manner. Maximum inhibitory effect was observed at 20 mM LiCl. Remarkably, motility inhibition by LiCl could be completely reversed by cAMP agonists, 2-chloroadenosine, and the protein phosphatase inhibitor calyculin A. Pretreatment of spermatozoa with these compounds also prevented the inhibitory effect of LiCl. The compound wortmanin had no effect on motility by itself or in combination with LiCl suggesting that PI-3 kinase pathway may not have a direct role on motility. Paradoxically, yet another inhibitor of PI-3 kinase, LY294002, reversed the inhibitory effect of LiCl on motility. We attribute this effect of LY294002 to an elevation of sperm cAMP levels rather than an inhibition of PI-3 kinase. Studies are underway to determine whether motility inhibition through inhibition of GSK-3 by lithium chloride involves changes in the activity of glycolytic enzyme and changes in serine phosphatase activities. Our data argue for an important role for GSK-3 in sperm function. (Supported by NIH HD38520)

1060

**Signaling through Ephrin-B3 Regulates Migration and Invasion of Glioma Cells**

M. Nakada, K. L. Drake, J. A. Niska, M. E. Berens; Neurogenomics, Translational Genomics Research Institute, Phoenix, AZ

Eph receptor tyrosine kinases and their ligands, ephrins, mediate neurodevelopmental processes such as boundary formation, axon guidance, vasculogenesis, and cell migration. To date, Eph receptor involvement in glioblastoma invasion has been demonstrated. Eph ligands, termed ephrins, are transmembrane proteins that bind to Eph receptors; binding leads to phosphorylation of an ephrin kinase domain, which prompts successive phosphorylation of many cytoplasmic substrate proteins. Here we determined the expression profiles of the ephrin-B family members in four glioma cell lines under migrating and non-migrating conditions. Ephrin-B3 mRNA was overexpressed in all four during migration (1.3-1.7 fold). Laser capture microdissection of invading glioblastoma cells revealed elevated ephrin-B3 mRNA (1.2-10.0 fold) in 8 out of 8 biopsy specimens. Forced expression of ephrin-B3 in two low expressor glioma cell lines (U87, T98G) stimulated cell migration and invasion concomitant with the tyrosine phosphorylation of ephrin-B3. In high expressor glioma cell lines (U251, SNB19), migration and invasion were accelerated by treatment with EphB2/Fc chimera. U251 and SNB19 cells transfected with ephrin-B3 siRNA showed decreased migration and invasion. Moreover, depletion of endogenous ephrin-B3 expression abrogated the increase of migration and invasion by EphB2/Fc, indicating that increased migration and invasion is dependent on ephrin-B3. Confocal imaging of U251 and SNB19 cells showed ephrin-B3 localized in lamellipodia of motile wild type cells, and revealed a significant morphological change and the absence of ephrin-B3 localization in cells transfected with ephrin-B3 siRNA. In human brain tumor specimens, ephrin-B3 expression and phosphorylation correlated with increasing grade of gliomas. These data suggests that dysregulation of ephrin-B3 expression or function may underlie glioma invasion.

1061

**Lysophosphatidic Acid (LPA) and Sphingosine 1-Phosphate (S1P) Markedly Regulate Tumor Cell Migration and Invasion of Three-Dimensional Collagen Matrices Through Membrane-Type Metalloproteinase 1 (MT1-MMP)**

K. E. Fisher, A. Pop, W. Koh, N. J. Anthis, W. B. Saunders, G. E. Davis; Pathology and Laboratory Medicine, Texas A&M Health Science Center, College Station, TX

Tumor cell navigation through three-dimensional (3D) extracellular matrices (ECMs) occurs via interactions between the cell and its environment. Here we describe new tumor invasion microassay systems that assess the ability of lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) to regulate tumor cell migration and invasion. In migration assays, most tumor cells migrated in response to LPA and were inhibited by S1P while HT1080 cells uniquely migrated in response to both lipids. Likewise, HT1080 cells invaded dramatically in 3D type I collagen invasion assays with either lipid. Treatment with synthetic matrix metalloproteinase (MMP) inhibitors or tissue inhibitors of metalloproteinases (TIMPs)-2, -3, and -4 strongly blocked HT1080 invasion but not migration suggesting a role for membrane-type MMPs (MT-MMPs) in invasion. siRNA targeting MT1-MMP markedly inhibited both S1P- and LPA-induced HT1080 invasion. Transfection of migratory, non-invasive HEK293 cells with MT1-MMP cDNA resulted in LPA-induced invasion of 3D collagen matrices. These data suggest that LPA effectively promotes the migration and invasion of multiple tumor cell types while S1P inhibits these processes in most cases. Thus, LPA markedly stimulates MT1-MMP dependent tumor cell invasion of 3D collagen matrices suggesting a role for coordinated signaling between LPA and/or S1P receptors and MT-MMPs during cellular invasive events.

1062

**SSX-new Molecular Target Regulating Cancer Invasion**

K. Itoh, K. Yoshioka; Biology, Osaka Medical Center for Cancer, Osaka, Japan

The SSX genes were initially identified as fusion partners to the SYT gene in human synovial sarcomas carrying a recurrent t (X; 18)(p11.2; q11.2) chromosomal translocation. Besides adult human testis, SSX genes were expressed at varying frequencies in a number of malignancies thereby categorized as cancer/testis antigens. Using nucleic acid sequence-based amplification, we have reported that the expression level in the malignant tumors (3.8+-1.4 copies/micro g of total RNA in log10 order) was significantly higher than that in the benign tumors (2.4+-0.5, $p<0.0001$). In order to examine the biological function of SSX, we firstly made stable transfectants with wild type SSX using human osteosarcoma cell line, SaOS2, which moderately expressed SSX. The expression of SSX was mainly localized in the nucleus with patched pattern. The SSX transfectants promoted colony formation in soft agar and tumor formation in nude mice, but showed little change in growth rate in culture. The transfectants also increased motility, chemotaxis and invasiveness using scratch wound assay and Boyden chamber assay. Since the C-terminal region of SSX strongly bound to Histone in the nucleus, we next made stable transfectants with C-terminal deletion mutants of SSX1, using human fibrosarcoma cell line, HT1080, which highly expressed endogenous SSX1. The transfectants showed marked stress fibers and focal adhesions, decreased motility, chemotaxis and invasiveness in vitro, and attenuated tumorigenesis in nude mice. Furthermore, the lowering of the endogenous expression of SSX1 in HT1080 cells by the treatment with specific siRNA markedly decreased chemotaxis, but did not affect cell proliferation. Collectively, these data suggested that SSX protein regulated several gene expressions resulting in leading to the tumor invasion, and could be a new molecular target under clinical setting.

1063

**Unraveling h-prune Function Together with Nm23 Family Protein in Breast Cancer induced Cell Motility and Metastases**

M. Zollo,[1] L. Garzia,[1] P. Carotenuto,[1] N. Marino,[1] C. Roma,[1] N. Tata,[1] A. M. Bello,[1] G. Vitale,[1] V. Maffia,[1] A. Amoresano,[2] N. Curtin,[3] A. Galeone[4]; [1]CEINGE, Naples, Italy, [2]Dipartimento di Chimica Organica e Biochimica, University Federico II, Naples, Italy, [3]Tyne Research Group, University of Newcastle, Newcastle, United Kingdom, [4]Dip. di Chimica delle Sostanze Naturali, University Federico II, Naples, Italy

Increase in cellular motility is one of the first events occurring during metastatic processes. We have demonstrated that overexpresssion of h-prune induces cells proliferation and we identified its cAMP phosphodiesterase activity effectively suppressed by dipyridamole, a phosphodiesterase inhibitor. High levels of h-prune protein versus low levels of nm23-H1 was found correlated to advanced breast cancer stage disease, thus being h-prune a new marker for diagnosis. The increase of h-prune phosphodiesterase activity resulted by protein overexpression and protein-protein interaction with nm23-H1 enhances cellular motility in breast cancer cells. Human nm23 genes display a regulative role in cellular proliferation, apoptosis, differentiation and tumor metastasis. Here we identified the molecular mechanism by which h-prune interacts with nm23 and how this complex takes part in the cellular motility network. The nm23 protein region is subjected to serine phosphorylation by Casein KinaseI. We show by spectrometry MALDI analyses, that the phosphorylation is occurring “in vivo” in a region of nm23 where serine residues of the protein are found phosphorylated. We found that IC261, a CKIδ-ε inhibitor, and a new competitive cell penetrating peptide impair nm23 specific region of phosphorylation and therefore the protein complex formation with h-prune. Additionally, we show that, IC261 and the specific peptide are both able to inhibit the h-prune pro-motility function, and the IC261 also cause h-prune relocation below the cytoplasm membrane (co-localizing with F-actin). These findings are presenting new challenges in the use of IC261 or the competitive peptide in the inhibition of cellular motility enhanced by h-prune-nm23 complex in breast cancer. References: D’angelo et al. *Cancer Cell.* 2004 D’Angelo and Zollo, *Cell Cycle*. 2004 Zollo et al. *Clinical Cancer Research.* 2005 Grants: AIRC-FIRC Fellowship (L.G), AIRC-FIRC 2005 grant, Bio))flag srl, Pula, Cagliari, Italy

1064

**Polycystin-1 Induces Cell Migration in MDCK Cells**

M. Boca,[1] D. Deflorio,[1] G. G. Germino,[2] A. Boletta[1]; [1]Functional Genomics, Dulbecco Telethon Institute (DTI)- DIBIT-HSR, Milano, Italy, [2]Division of Nephrology, Johns Hopkins University School of Medicine, Baltimore, MD

Autosomal Dominant Polycystic Kidney Disease, one of the most common inherited disorders, is characterized by cyst formation in the kidney. Mutations of PKD1 are responsible for ~85% of all cases. Polycystin-1 (PC-1), its gene product, is a large (520kDa) non-tyrosine kinase receptor involved in cell-cell/matrix interactions as well as in mechanosensation on the primary cilium. We have shown that PC-1 expression in renal epithelial cell (MDCK, Madin Darby Canine Kidney), determines reduced growth rates, resistance to apoptosis and spontaneus tubologenesis in 3D collagen gels. We now find that PC-1 expressing cells have a scattered phenotype as compared to controls that grow in clusters. Staining with rodamine-labelled phalloidin revealed profound cytoskeletal re-arrangements in these cells as compared to controls, suggestive of an acquired migratory phenotype. We therefore perfomed time-lapse videomicroscopy of wound healing assays to assess the migratory properties of PC-1 expressing cells. Interestingly, the control cells move forward as a compact unit, the edge remaining attached to adjacent cells. In contrast, PC-1 expressing cells start pulling away from the edge as individual cells and acquire a polarized migratory phenotype. In addition, the rate of migration in these cells is considerably higher (14.62 ± 2.38 μm/h in PC-1 expressing cells vs 9.25 ± 2.71 μm/h in controls). Concomitantly, E-cadherin and β-catenin are quickly internalized. In search for the molecules that could mediate cell migration and tubulogenesis in our model system we have found that Phosphatidylinositol-3-kinase (PI3k) as well as Glycogen Synthase Kinase 3β (GSK3β) seem to be implicated, although in different aspects of the phenotypes. Furthermore, we find activation of the small GTPase cdc42 during wound healing assays in PC-1 positive cells, but not in the controls. We are currently investigating the role played by these different molecules in Polycystin-1 mediated cell migration.

1065

***Helicobacter pylori* CagA Induces AGS Cell Elongation Independent of Cdc42 and Rac1**

K. M. Bourzac, K. J. Guillemin; Institute of Molecular Biology, University of Oregon, Eugene, OR

*Helicobacter pylori* is the causative agent of gastric diseases including duodenal ulcers and gastric cancer. A more severe disease outcome is associated with strains harboring the *cag* pathogenicity island whose genes include components of a Type IV secretion system and its secreted substrate, CagA. Once delivered into host cells, CagA is tyrosine phosphorylated by Src-family kinases and then binds to and activates SHP-2 phosphatase and other components of the c-Met receptor tyrosine kinase pathway. This inappropriate signaling is thought to lead to cancer

development. The gastric adenocarcinoma cell line AGS respond to CagA-induced signaling by undergoing pronounced cellular elongation in tissue culture. While recent studies have identified key steps in early CagA signaling events, little is known about downstream molecules important for initiation of cell shape changes. The Rho GTPases Cdc42 and Rac1 are key mediators of actin dynamics, and there has been speculation that they could play an important role in the CagA phenotype. To test this hypothesis, AGS cells were transiently transfected with dominant negative alleles of Cdc42 or Rac1 and then challenged with *H. pylori* infection. We found that neither of these constructs blocked AGS cell elongation. Cdc42 effector protein 2 (CEP2), a downstream effector of Cdc42 that is upregulated in AGS cells during infection, was also found to play no role in the elongation phenotype. Further, we were unable to detect activation of Cdc42 or Rac1 during an infection time course using GST-CRIB affinity pulldown experiments. We also found that the localization of the cellular population of Cdc42 and Rac1 is not changed during infection. Therefore, we conclude that CagA-induced cell elongation is independent of Cdc42 and Rac1.

## Cytoskeletal Organization I (1066-1089)

1066

### CD28 Initiates Polarization of the Microtubule Organization Center and Cytolytic Granules to the Synapse by Inducing Phosphorylation of ERK2

X. Chen, D. Allan, K. Krzewski, J. L. Strominger; Molecular and Cellular Biology, Harvard University, Cambridge, MA

Activation of natural killer (NK) cell cytotoxicity requires adhesion and formation of a conjugate with a susceptible target cell, localized actin polymerization, and polarization of cytolytic granules for targeted release. Previously PI3K-mediated ERK activation was shown to be required for cytolytic granule translocation, and thus necessary for NK cell cytotoxicity (*Djeu Y.J. et al. Clinic cancer research, V8:636,2002*). Here, the mechanism of the phenomenon has been examined in detail. The PI3K/ERK pathway is shown to modulate microtubule rearrangement. Specifically, activated ERK2 (but not ERK1) participates in NK cell cytotoxicity by contributing to the polarization of the microtubule organization center (MTOC) to the NK cell immune synapse (NKIS). ERK is observed to directly interact with microtubule filaments. Actin polymerization in the NKIS and NK cell/target cell conjugate formation is not affected by blocking ERK activation. Using the YTS NK cell line model, CD28 signaling plays an essential and sufficient role in stimulating NK cell cytotoxicity, while LFA-1 signaling is normally required but not sufficient. Interestingly, CD28 signals MTOC polarization through sustained ERK2 phosphorylation. On the contrary, LFA-1 ligation transduces a transient ERK2 activation and fails to polarize the MTOC. Rather, LFA-1 signals initiate actin polymerization and conjugate formation, while CD28 does not. Thus, in YTS cells, these two receptors appear to play distinct functional roles, each essential for formation of the NK immune synapse, regulating different cytoskeletal processes, and differing in their pattern of ERK2 activation.

1067

### The Regulation of Immune Cell Function by the Rho GTPase Rif

S. L. Passey, S. Pellegrin, H. Mellor; Biochemistry, Univerisity of Bristol, Bristol, United Kingdom

Rho family GTPases are critical regulators of the actin cytoskeleton, and play important roles in regulating cell shape and motility. We have shown that the novel Rho GTPase family member Rif (Rho In Filopodia) induces the formation of long actin-based structures called filopodia when expressed in a number of mammalian cell types. We find that it functions independently of the classical filopodia-inducing Rho GTPase Cdc42 as well as the downstream signalling components of this pathway WASP and Arp2/3, instead Rif induces filopodia formation the actin-nucleating formin mDia2. Thus Rif represents an alternative route to filopodia formation which allows the cell to generate subtly different filopodia-like protrusions to perform different roles. We are particularly interested in the physiological role for Rif and Rif-induced filopodia in mammalian cells. Microarray data and western blotting have revealed that Rif is highly expressed in a subset of immune cells, suggesting that it may play an important role in immune function. Overexpression of Rif and constitutively active Rif mutants in these cells results in dramatic actin rearrangement. We are currently investigating the regulation of the actin cytoskeleton by Rif in immune cells and hypothesise that Rif plays an important role in regulating the essential changes in morphology that underpin immune cell function and the generation of an efficient immune response.

1068

### The Rho Family GTPase Rif Induces Filopodia through mDia2

S. Pellegrin, H. Mellor; Biochemistry, University of Bristol, Bristol, United Kingdom

The Rho GTPases RhoA, Rac1 and Cdc42 are well-characterised regulators of the actin cytoskeleton but little is known about the other 19 mammalian Rho GTPases. We have isolated a novel Rho-family GTPase, Rif (Rho in filopodia), which is present in chordates including ascidians. When transiently expressed in mammalian cells, Rif is localised at the plasma membrane and induces the formation of long, thin, actin-rich projections called filopodia. The Rho GTPase Cdc42 is a key mediator of filopodia formation. Cdc42 may generate filopodia by regulating CRIB motif-containing effectors including WASP proteins, IRSp53 and mDia2. We have shown that Rif induces filopodia independently from Cdc42 and does not bind CRIB motifs. Rif interacts with the formin mDia2 and mDia2 is required for Rif-induced filopodia formation. The CRIB motif of mDia2 is not required for its interaction with Rif and the filopodia generated by Cdc42 are morphologically different to those induced by Rif. Thus Rif and Cdc42 represent two distinct routes to the induction of filopodia, producing structures with both shared and unique properties.

1069

### Chemical Genetic Analysis of the Orb6 Kinase Pathway in *S pombe*

S. M. Nicholls,[1] D. Wiley,[1] M. Tress,[1] K. Shah,[2] F. Verde[1]; [1]Pharmacology, University of Miami, Miami, FL, [2]Chemistry, Purdue University, West Lafayette, IN

We have previously identified Orb6, a serine/threonine kinase in *Schizosaccharomyces pombe*, which functions in the control of cell morphology. Orb6 is conserved in higher eukaryotic organisms, and is related to mammalian NDR1 and NDR2 kinases, which have a role in tumorigenesis and in the control of cell proliferation. To explore the physiological role of Orb6 kinase activity, we have employed a chemical genetic approach involving site-directed mutagenesis of the kinase active site, which creates a new pocket where specific compounds bind to inhibit kinase activity

(Alaimo *et al*., 2001). Therefore, we have chemically inhibited the function of the Orb6 kinase *in vivo* by altering the ATP binding site so that it can accept the kinase inhibitor 1-NA-PP1 (1-naphthylmethyl-pyrazolo pyrimidine-1). We found that when the Orb6 kinase mutant (*orb6-as2*) cells are exposed to the inhibitor, the actin cytoskeleton is rapidly altered. Actin cables quickly disappear and actin patches become uniformly distributed throughout the cell cortex. Furthermore, we found Orb6 kinase inhibition does not affect actin ring assembly during mitosis in synchronized cells, however it prevents post-telophase actin reorganisation and cell separation. These studies suggest that Orb6 kinase activity controls actin cable and actin patches dynamics, and promotes actin reorganization after mitosis. To identify potential effectors of the Orb6 pathway, we have employed a number of techniques, including Mass Spectometry analysis of the Orb6-associated protein complex and 2-hybrid and genetic screens. Several proteins that are potential effectors of the Orb6 pathway have been identified.

1070

### Two Distinct Modes of Epithelial Repair

M. Tamada,[1] T. Perez,[2] W. J. Nelson,[2] M. P. Sheetz[1]; [1]Biological Sciences, Columbia University, New York, NY, [2]Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA

Morphogenesis involves the bending and folding of epithelial sheets to mold the form of the embryo. Non-muscle myosin II is known to generate forces needed for tissue remodeling during a variety of developmental processes in many organisms. However, the regulatory mechanisms for its polarized assembly and activation are poorly understood. We laser-ablated cells in a confluent monolayer of MDCKII cells expressing EGFP-tagged myosin regulatory light chain (MLC) and found that MLC accumulated at the apical and basal tips of the lateral membranes of cells exposed to the ablated cells. We identified two types of separately regulated cell movements during extrusion of ablated cells: 1) the extension of a thin basal cell membrane accompanied by a basal accumulation of MLC, and 2) the shortening of the lateral cell membrane exposed to the ablated cells accompanied by an apical accumulation of MLC, which formed a ring-like structure where ZO-1 was located. Rho-kinase was also recruited to the same site with apical MLC and was found to be required for MLC accumulation and the proper changes in cell shape. These results from living cells show how cells re-organize in response to morphological changes, and suggest that the tight junction includes the machinery to stimulate the formation of myosin fibers required for changes in epithelial cell shape.

1071

### Fibroblasts Lacking Zyxin Exhibit Increased Motility and Deficits in Actin Stress Fiber Remodeling

L. M. Hoffman, C. C. Jensen, M. Yoshigi, M. C. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Integrin receptors and associated proteins link the extracellular matrix to the intracellular actin cytoskeleton at specialized structures called focal adhesions. To determine the cellular function of the focal adhesion protein zyxin, we isolated and characterized fibroblasts from zyxin-null mice. Compared to wild-type fibroblasts, zyxin-null fibroblasts were more migratory in monolayer wound assays. Transwell invasion assays through Matrigel also showed enhanced migration of zyxin-null fibroblasts, a characteristic that was abrogated by the reintroduction of GFP-zyxin into the zyxin-null fibroblasts. Molecular differences between wild-type and zyxin-null cells were explored by using anti-phosphotyrosine antibodies on immunoblots. A tyrosine-phosphorylated protein which was different between wild-type and zyxin-null fibroblasts was determined to be the actomyosin regulator caldesmon. To challenge the actin cytoskeleton, fibroblasts were treated with jasplakinolide, a membrane permeable agent which induces actin assembly and stress fiber reinforcement. Wild-type cells developed thick stress fibers in response to jasplakinolide, but cells lacking zyxin were deficient in this response. Reintroduction of GFP-zyxin restored the ability of the cells to reinforce their cytoskeletons, demonstrating that the defect was directly related to zyxin function. In wild-type cells exposed to jasplakinolide, zyxin dramatically mobilized from focal adhesions to actin filaments. The actin assembly regulator VASP also redistributed to actin stress fibers in response to jasplakinolide and this response required zyxin. Zyxin may contribute to stress fiber reinforcement by localizing Ena/VASP proteins and perhaps influencing their activity. We hypothesize that zyxin, in cooperation with Ena/VASP proteins and caldesmon, influences cell motility and the actin cytoskeleton.

1072

### Brush Border Structure Defects in I-Plastin Deficient Mice

E. M. S. Grimm-Günter,[1] S. Ramos,[1] N. Smyth,[1] C. Revenu,[2] I. Hurbain,[2] G. Raposo,[2] S. Robine,[2] F. Rivero[1]; [1]Center for Biochemistry, Medical Faculty, University of Cologne, Cologne, Germany, [2]UMR 144, Institut Curie, Paris, France

Plastin (or fimbrin) belongs to a subclass of cytoskeletal proteins known as actin-bundling proteins. They consist of two actin-binding domains in tandem preceded by two calcium-binding EF-hands. Plastins are conserved from lower eukaryotes to humans. In mammals three different isoforms are expressed in a cell-type specific manner. I-plastin is specifically expressed in the small intestine, colon and kidney, whereas the isoforms L- and T-plastin are expressed in leukocytes and in all other tissues, respectively. Generally plastins are located in focal adhesions, ruffling membranes, lammellipodia and filopodia. I-plastin localizes in specialized surface structures with highly ordered microfilament bundles such as microvilli and stereocilia. We have generated knockout mice lacking I-plastin. We verified the recombination event by Southern, Northern and Western analyses. I-plastin deficient mice showed no overt phenotype at the whole animal level: growth rate, reproductive rate and litter size were normal. Antibodies specific for the plastin isoforms T- and L-plastin were used to demonstrate that lacking of I-plastin is not compensated by another plastin isoform. Transmission electron microscopy studies revealed morphological differences between wildtype and I-plastin deficient mice in the microvilli of the small intestine. Brush border preparations of knockout mice are sensitive and seem to degrade easily. In immunofluorescence studies of intestinal mucosa cryosections decreased actin staining of the apical membrane was observed. Another abundant actin cross-linker of the brush border, villin, was found to be absent from the apical membrane. I-plastin seems to be an important regulator of the morphological structure and stability of microvilli. Further studies will allow investigating the contribution of this protein to the organization of the intestinal brush border barrier and its role during infectious processes.

1073

### Intracellular Response to Shear Flow is Mediated by Rho-kinase

J. S. Lee,[1] P. Panorchan,[1] Y. Tseng,[2] D. Wirtz[1]; [1]Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, [2]Chemical Engineering, University of Florida, Gainsville, FL

Cells *in vivo* are often subjected to mechanical shear stresses which have been shown to play important roles in various physiological and pathological processes. However, remarkably little is known about whether and how the cytoskeleton of an adherent cell adapts its mechanical properties in response to fluid shear stresses. Here, by modifying the method of intracellular microrheology (ICM), we monitor the micromechanical response of the cytoskeleton of single cells exposed to flow. ICM reveals that the formation of contractile actin stress fibers and focal adhesions induced by shear in serum-starved Swiss 3T3 fibroblasts mediates a remarkable 25-fold and 4-fold increase in cytoplasmic elasticity and viscosity, respectively. In contrast, our previous study shows that the cytoskeleton of the same cells treated with LPA undergoes only highly transient stiffening of much lower amplitude, despite the formation of long-lived stress fibers and focal adhesions. Pretreatment with either an actomyosin interaction inhibitor or Y-27632, a specific Rho-kinase inhibitor, prevents the formation of stress fibers and eliminates shear-induced cell stiffening. Together these results reveal that adherent cells subjected to fluid shear stress strengthen their cytoskeleton and that biochemical and biophysical stimuli may elicit the formation of seemingly similar cytoskeleton organizations, but qualitatively different micromechanical responses. This study also identifies Rho-kinase as a key factor in the mechano-transduction pathway that controls the cytoskeleton mechanical response of cells to shear.

1074

**Analysis the Mechanism during Otic Placode Invagination**

X. Sai, R. Ladher; Sensory Development, RIKEN CDB, Kobe, Japan

Morphological development of the inner ear begins with formation of the otic placode. Our scanning electron microscope show that the otic placode of stage HH9 (7 somites stage) chick embryo can be observed as a thickened otic primordia on the surface ectoderm. This thickening region invaginates gradually, eventually sealing itself up to form an otocyst at HH17 (64 hours). The mechanisms of this complicated cellular rearragement remain unknown. Using light microscopy, we observed the cells of the otic placode. Cells appear to change shape as the otic placode invaginates, from columnar to a more wedge-shaped appearance. We were interested in further characterizing these cells and used transmission electron microscopy to investigate the intracellular structures. TEM results show unique cytoskeletal structure. Firstly, in a few cells nuclei were displaced basally and in these cells microtubules were located apical to the nucleus. Secondly, F-actin fibers located at the apex of the cells seemed to function to contract the apical membrane of the cells. Thirdly, intermediate filaments were observed around the nuclei of several cells located centrally within the otic placode. Treatment of the otic placode with notacozone and cytochacin D inhibited the closure of the otocyst. We are currently determining the nature of the extrinsic signals and the mechanism of nuclear migration and establishing causal relationships between cellular changes and morphological tissue rearrangement. Our studies imply that microtubules and intermediate filaments play a role in directing basal localization of the nuclei of several cells and that F-actin is important for the purse-string constriction of the peripheral cells. These and other, as yet uncharacterized cellular properties drive the sheet of otic placode cells to invaginate, change morphology into a hollow sphere and become internalized into the head mesenchyme of the embryo.

1075

**Role of PTP-PEST in the Regulation of Phosphorylation of WASP and Resulting Osteoclast Bone Resorption**

M. Chellaiah,[1] D. Kuppuswamy,[2] C. Willey[2]; [1]Department of BMS, University of Maryland, Baltimore, MD, [2]Gazes Cardiac Research Institute, Medical University of S.Carolina, Cardiology Division, Charleston, SC

We have previously shown that osteopontin (OPN)/αvβ3-mediated signaling facilitates WASP to interact with Arp2/3 complex and thereby enable the latter to promote actin nucleation, polymerization and ring formation in osteoclasts. Actin ring formation is a prerequisite for efficient bone resorption in osteoclasts. Despite success in identifying the role of kinases such as, Src, PYK2 and FAK in actin ring formation, the potential target protein of these kinase(s) is poorly understood. WASP phosphorylation was shown to be mediated by several kinases by studies in different cell systems. Phosphorylation state of WASP and WASP interacting protein(s) was determined in osteoclasts treated with OPN or expressing either constitutively active (CA-Src) or kinase negative- Src (KN-Src) by adenoviral- mediated delivery. *In vitro* kinase assay analysis demonstrated an increase in phosphorylation of Src, PYK2, WASP, and three other proteins with MW of 100-110kDa, 120-130kDa, 35-40kDa in OPN-treated and in osteoclasts expressing CA-Src but not in KN-Src. Protein with MW of p100-110 was confirmed as PTP-PEST by Western analysis. Confocal studies revealed that WASP and PTP-PEST form a complex and are colocalized in OPN-treated osteoclasts. Measurement of WASP-associated PTP-PEST phosphatase activity exhibited decreased activity levels both in OPN-treated and CA-Src expressing osteoclasts. Furthermore, OPN stimulation resulted in serine phosphorylation of WASP-associated PTP-PEST. Such increased serine phosphorylation of PTP-PEST was accompanied by an increased tyrosine phosphorylation of WASP and other associated signaling proteins. Experiments with PTP-PEST inhibitor and SiRNA to PTP-PEST further confirmed the involvement of PTP-PEST in the WASP-Arp2/3 complex, actin ring, and bone resorption. Our observations suggest that WASP, which is identified in the actin ring of osteoclasts, associates with Src, PYK2, and PTP-PEST. PTP-PEST and tyrosine kinase(s) coordinate the formation of WASP-Arp2/3 complex and the actin ring

1076

**Coronin 2A Expression Regulates Slingshot-1L Activity**

T. W. Marshall, J. E. Bear; Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC

Coronins are a conserved family of actin cytoskeleton regulators found in all eukaryotes. Coronin 2A and 2B represent a subclass of Coronins (type II) that are found only in vertebrates. These genes are expressed in a restricted manner across tissues. Coronin 2A is highly expressed in the testis, ovaries, and uterus and moderately expressed in the brain and kidneys, while Coronin 2B is highly expressed in the brain with moderate expression in the kidneys. Overexpression of Coronin 2A in Rat2 fibroblasts induces the formation of Cofilin-Actin rods and reorganizes F-actin stress fibers. The stress fibers appear to be pushed to the periphery of the cell and become thicker. This stress fiber phenotype is similar to the phenotype observed when Cofilin activity is inhibited by either overexpressing Lim Kinase or phosphatase-dead Slingshot 1-L (SSH1L). Coronin 2A and SSH1L reciprocally coimmunoprecipitate suggesting that these proteins are in a complex together in cells. By immunofluorescence, the two proteins also strongly colocalize along stress fibers. Slingshot also localizes to the leading edge of the lamellapodia, but Coronin 2A does not. Coronin 2A overexpression increases P-Cofilin levels indicating that Coronin 2A may be affecting Slingshot phosphatase activity. The interaction between Coronin 2A and Slingshot is dependent upon the coiled-coil domain of Coronin 2A. Upon expression of a coiled-coil deletion mutant of

Coronin 2A, SSH1L becomes more enriched at the leading edge, leading to the hypothesis that Coronin 2A may be sequestering Slingshot away from the leading edge. The interaction between Coronin 2A and Slingshot may play a key role in regulating Cofilin activity. Currently, we are determining the in vivo function of type II Coronins and the importance of Coronin 2A on regulating the phosphorylation status of Cofilin.

1077

**Interaction of Smitin Zq Domain with Alpha-actinin Central Rod Spectrin Repeat-like R2-R3 Loops**

R. J. Chi, S. G. Olenych, T. C. S. Keller; Biological Science, Florida State University, Tallahassee, FL

Alpha-actinin interacts with the myosin-binding protein titin to provide an important structural linkage in the Z-line of striated muscle sarcomeres. A similar interaction may contribute to smooth muscle contractile apparatus organization. We previously discovered a titin-like protein - smitin - in smooth muscle cells and mapped smooth muscle alpha-actinin binding sites for native smitin to the central rod R2-R3 spectrin repeat-like and C-terminal domains, sites that are analogous to those in striated muscle alpha-actinin that bind respectively to titin Zq and Z-repeat domains. Using RT-PCR analysis of RNA from rat aortic smooth muscle A7r5 cells and human carotid artery to investigate whether smitin contains Z-repeat and Zq-like domains, we have found products containing sequences identical to exon sequences of the titin gene that encode Z-repeat and Zq domains. A polyclonal antibody raised against an expressed protein encoded by this sequence stained the contractile apparatus of the A7r5 cells in a punctate pattern similar to that for smitin localization. We used the expressed Z-repeat-Zq peptide to test the hypothesis that loops in the R2 and R3 spectrin repeat-like domains, which are modeled to lie in proximity on the surface of the central rod and are highly conserved between smooth and striated muscle titins, constitute the binding site for the Zq domain. We found that a T425A mutation in the R2 loop and a D586A mutation in the R3 loop both decrease binding to the Zq domain in GST-pulldown and solid phase binding assays. These results suggest that smitin contains Z-repeat and Zq domains encoded by the known titin gene and that conserved surface loops from the R2 and R3 spectrin repeat-like domains form the binding site of smooth and striated muscle alpha-actinins for smitin and titin Zq domains.

1078

**Comparing the Effects of siRNA vs Pharmacological Inhibitor of GTPase Kinases**

H. Darenfed,[1] B. Dayanandan,[1] A. Fournier,[2] C. A. Mandato[1]; [1]Anatomy & Cell Biology, McGill University, Montreal, PQ, Canada, [2]Neurology & Neurosurgery, McGill University, Montreal, PQ, Canada

Rho- family GTPases are key regulatory molecules that link extracellular ligands to the organization of the actin cytoskeleton. They regulate many cellular functions including cytokinesis, cell adhesion, motility, stress fiber formation and neuronal growth cone extensions. The Rho protein acts as a switch by cycling between a GTP bound active form and GDP bound inactive form. Once activated it binds to a wide range of effectors to stimulate downstream signaling pathways. These effectors include the ROCK family members, ROCK1 and ROCK2, which regulate multiple downstream substrates to modulate cellular responses. The compound, Y-27632, has been extensively used as a ROCK2 inhibitor for studying the role of ROCK2 in actin/ myosin organization and cytokinesis. However, the mechanistic relevance of this inhibition has not been explored. This is an important question when considering the mechanism of Y-27632 action on various cellular processes. In this study we investigated the role of ROCK2 in fibroblasts and neuronal primary cultures using small interfering RNAs (siRNA). Knockdown of ROCK2 expression levels mediates alterations in actin organization. On the other hand treatment with Y-27632 induces the formation of thin actin extensions and reduces the expression of stress fibers. These results suggest that Y-27632 compound may affect other kinases regulating actin organization during different cellular activities.

1079

**Regulation of Signaling Pathways by Myelin Inhibitors**

J. M. Robens, E. Manser; Institute for Molecular and Cellular Biology, Singapore, Singapore

Neurons in the CNS have the capacity to regenerate once damaged but are inhibited in part by myelin-derived inhibitors such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp). These inhibitors activate a trimeric receptor complex on neuronal membranes and regulate the activation of small GTPases of the Rho subfamily to remodel the actin cytoskeleton leading to growth cone collapse. The mechanism by which this trimeric complex, composed of the Nogo66-receptor (NgR), p75 neurotrophic receptor (p75NTR)/TROY, and Lingo-1, regulates these GTPases still remains elusive. Since the receptors in this complex do not have intrinsic enzymatic activity, this suggests that the recruitment of proteins to the activated receptor complex is crucial in the regulation of these signaling pathways. We have recently identified potential partners that are recruited to the activated NgR-receptor complex. Our current research is now focused on the mechanism by which these recruited proteins regulate the activation of Rho family members and subsequently the remodeling of the actin cytoskeleton and inhibition of neurite extention. This research will provide useful insights into the regulation of signaling pathways by myelin-derived inhibitors and how they may be manipulated therapeutically to treat neurological disorders.

1080

**Centralspindlin Regulates ECT2 and RhoA Accumulation at the Equatorial Cortex during Cytokinesis**

Y. Nishimura, S. Yonemura; RIKEN, Center for Developmental Biology, Kobe, Japan

During determination of the cell division plane, an actomyosin contractile ring is induced at the equatorial cell cortex by signals from the mitotic apparatus and contracts to cause cleavage furrow progression. Although the small GTPase RhoA is known to regulate the progression probably by controlling actin filament assembly and enhancing actomyosin interaction, involvement of RhoA in division plane determination has been largely unknown. In this study, using a Trichloroacetic Acid (TCA) fixation protocol we recently developed, we show that RhoA accumulates at the equatorial cortex before furrow initiation and continues to concentrate at the cleavage furrow during cytokinesis. These observations suggest that RhoA accumulation at the equatorial cortex is involved in formation of the contractile ring during the early stage of cytokinesis. We also demonstrate that both Rho activity and microtubule organization are required for RhoA localization and proper furrowing. Selective disruption of microtubule organization revealed that both astral and central spindle microtubules could recruit RhoA at the equatorial cortex. To elucidate the molecular mechanism of RhoA localization at the equatorial cortex, we tested several candidate proteins that might connect microtubules and RhoA activity. We find that centralspindlin and ECT2, that have been known to form a complex on the central spindle microtubules, are required

for RhoA localization and furrowing. RNAi experiments indicate that centralspidlin regulates RhoA localization through regulation of ECT2 localization. Positional information for division plane determination from microtubules appears to be transmitted to the cell cortex to organize actin cytoskeleton through a mechanism involving these proteins.

1081

**Timed Study of S-crystallin Translation in Light- and Dark-adapted Octopus Retinas**

T. Taylor, S. Kelly, L. J. Robles; Biology, California State University, Dominguez Hills, Carson, CA

Morphological differences exist between light- and dark-adapted octopus retinas with the rhabdomeric microvillus actin cytoskeleton undergoing dramatic reorganization in conditions of light vs. dark. Recent studies showed that S-crystallin is present in significantly greater amounts in dark-adapted octopus retinas and it binds to F-actin, while in light-adapted retinas, S-crystallin is greatly reduced with no apparent F-actin binding. We are investigating whether the presence of S-crystallin is time sensitive; that is, if translation of S-crystallin varies with increasing time in the light or dark. Octopi were either light- or dark-adapted 3-4 hours after which the eyecups of one octopus were dissected for controls. The remaining octopi were transferred to the opposing lighting condition and sacrificed at 15 minute intervals for a period of two hours. At each time point, the octopus was anesthetized on ice, the eyes dissected, the retinas homogenized in phosphate buffer, and protein isolated. Concentrations of the retinal homogenates were determined and equal amounts of protein from each interval were loaded onto SDS gels. The proteins were separated and translational differences in S-crystallin quantified. Translational differences in actin were also analyzed. Preliminary results indicate initially increased levels of S-crystallin, from 5.36% to 10.4%, in the first 15 to 30 minutes of animals moved to the dark after which the levels decrease slightly and remain constant in the second hour. Preliminary results from animals moved to the light indicate more constant but cyclically fluctuating levels of S-crystallin. Increases in S-crystallin during the first 30 minutes in the dark and cyclical levels in the light suggest that the involvement of S-crystallin in the reorganization of the rhabdomeric actin cytoskeleton may be time sensitive. NIH GM08156/62252

1082

**MLCK/actin Interaction in the Contracting A7r5 Smooth Muscle Cell**

S. E. Thatcher, G. L. Wright; Physiology, Joan C. Edwards School of Medicine, Huntington, WV

Recent biochemical data indicates that MLCK could contribute to actin cytoskeletal structure. Since dynamic remodeling of actin to different contractile agonists has been reported, it was of interest to examine MLCK/actin interaction during contractile reorganization of the cytoskeleton. Fluorescent resonance energy transfer (FRET) was utilized to evaluate MLCK association with α- and β-actin in cells contracted with phorbol 12,13 dibutyrate (PDBu) or by addition of the calcium ionophore A23187. Results showed a significant increase (70%) in MLCK/α-actin interaction with PDBu stimulation. By comparison, the interaction between β-actin and MLCK was unchanged after treatment with PDBu. Addition of the calcium ionophore A23187 resulted in a significant increase in the association of MLCK with α-actin (83%) but not β-actin. These results suggest MLCK recruitment and increased interaction with the α-actin cytoskeleton during PDBu- and A23187-induced contraction. This dynamic interaction could be important in optimizing contractile mechanical advantage and/or contribute to actin/myosin reorganization during contraction.

1083

**A PAK4/alphaPIX Complex Regulates Podosome Size and Number in Macrophages**

S. Linder,[1] A. Gringel,[1] D. Walz,[1] G. Rosenberger,[2] K. Kutsche,[2] A. Minden[3]; [1]LMU, Munich, Germany, [2]UKE, Hamburg, Germany, [3]Columbia University, New York, NY

Podosomes are actin-rich adhesion structures typically found in monocytic cells. Major modes of podosome regulation include RhoGTPase signaling and actin regulatory pathways. However, it is not clearly understood how RhoGTPase-triggered signals induce highly localized changes in podosome formation and dynamics. Here, we show that the RhoGTPase effector PAK4, a member of the p21 associated kinase family, and the PAK interactive exchange factor alphaPIX, are central to localized podosome regulation in primary human macrophages. Importantly, part of the cellular pool of PAK4 was found to be associated with podosomes, while expression of PAK4 deletion/truncation mutants as well as transfection of specific siRNA resulted in reduced numbers of podosomes per cell. Moreover, expression of constitutively active or inactive PAK4 mutants enhanced or reduced, respectively, the size of individual podosomes. Similar to the results gained with PAK4, cellular/overexpressed PIX was shown to be closely associated with podosomes, and expression of alphaPIX mutants strongly influenced the ability of cells to form podosomes. As PAK4 and alphaPIX interact in cell lysates, we propose that a complex of both proteins regulates podosome size and number in these cells.

1084

**Epiplakin Is Dispensable for Skin Barrier Function and for Integrity of Keratin Network Cytoarchitecture in Simple and Stratified Epithelia**

D. Spazierer,[1] P. Fuchs,[1] S. Reipert,[1] M. Schmuth,[2] H. Lassmann,[3] G. Wiche[1]; [1]Molecular Cell Biology, University of Vienna, Vienna, Austria, [2]Department of Dermatology and Venereology, Medical University of Innsbruck, Innsbruck, Austria, [3]Center for Brain Research, Medical University of Vienna, Vienna, Austria

Epiplakin, a giant epithelial protein of >700 kDa, belongs to the plakin family of cytolinker proteins. It represents an atypical family member, however, as it consists entirely of plakin repeat domains but lacks any of the other domains commonly shared by plakins. Hence, its putative function as a cytolinker protein remains to be shown. To investigate epiplakin's biological role we generated epiplakin-deficient mice by gene targeting in embryonic stem cells. Epiplakin-deficient mice were viable and fertile, without developing any discernible phenotype. Ultrastructurally, their epidermis revealed no differences compared to wild-type littermates, and cornified envelopes isolated from skin showed no alterations in shape or stability. Furthermore, neither embryonal formation nor later function of the epithelial barrier were affected. In primary cultures of epiplakin-deficient keratinocytes the organization of actin filaments, microtubules, and keratin networks were found to be normal. Similarly, no alterations in keratin network organization were observed in simple epithelia of small intestine and liver, or in primary hepatocytes. We conclude that, despite epiplakin's abundant and highly specific expression in stratified and simple epithelia, it's absence in mice does not lead to severe skin dysfunctions, nor has it detectable consequences for keratin filament organization and cytoarchitecture of cells.

1085

**The Cytoskeleton-Associated Protein Palladin Interacts with Eps8, a Barbed-End Capping Protein**

S. M. Goicoechea,[1] A. Disanza,[2] G. Scita,[2] C. Otey[1]; [1]Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]IEO, European Institute of Oncology, IFOM, The FIRC Institute for Molecular Oncology, Milan, Italy

The cytoskeleton-associated protein palladin has been shown to play an important role in organizing the actin cytoskeleton. Palladin may function as a critical cytoskeletal scaffold, as it interacts with diverse actin-regulating proteins: profilin, members of the Ena/Mena/VASP family of proteins, the filament cross-linking protein, alpha-actinin, and LASP-1, an actin-associated LIM and SH3 domain-containing protein. We reasoned that palladin was likely to have additional binding partners, and we employed a yeast two-hybrid approach to identify them. Here, we describe a novel interaction between palladin and Eps8, a receptor tyrosine kinase (RTK) substrate that participates in the activation of the Rac-specific guanine nucleotide-exchange function of Sos1, thereby regulating actin remodeling by RTKs. Eps8 was identified in a yeast two-hybrid screen, using full-length palladin as bait. The interaction was confirmed biochemically, using *in vitro* and *in vivo* assays. Binary assays in yeast and immunoprecipitation assays from lysates of cultured cells showed that the interaction is direct and specific. The interacting domains were mapped to the proline-rich region of palladin and the aa 350-530 region of Eps8. The two proteins co-localize extensively in stress fibers, cell-cell junctions and also in the transient, circular, dorsal ruffles that form in response to growth factor stimulation. Together, these data provide strong evidence for a direct and specific interaction between palladin and Eps8. Our data suggest that these two proteins act together in stress fibers and in the rapid and transient remodeling of the actin cytoskeleton that promotes the formation of highly dynamic membrane protrusions in response to growth factor treatment.

1086

**Characterization of Cellular Titin in Human Megakaryocyte and Mouse Fibroblast Cell Lines**

P. J. Cavnar, S. Olenych, T. C. Keller; Department of Biological Sciences, Florida State University, Tallahassee, FL

We previously discovered a large titin-like protein - c-titin - in chicken epithelial brush border and human blood platelet extracts that binds alpha-actinin and organizes arrays of myosin II bipolar filaments in vitro. We have investigated the molecular relationship between c-titin and striated muscle titin by RT-PCR analysis of total RNA from human megakaryoblastic (CHRF-288-11) and mouse fibroblast (3T3) nonmuscle cells. Amplified products obtained contain sequences identical to known titin gene exon sequences that encode parts of the Z-line, I-band, PEVK domain, A-band, and M-line regions of striated muscle titins. Products containing Z-line exons encode the Z-repeat domains Zr1-3 and 7 and the Zq domain, which bind alpha-actinin in striated muscle titins. I-band region sequences encode the N2A exons, but none of the exons distinctive for the N2B or Novex I and II isoforms. Products from the A-band and M-line regions contain the long titin gene exon that encodes an Ig-FN3 domain superrepeat region important for myosin filament binding and the exon that encodes the kinase domain. We found two variants containing PEVK-encoding exons not known to be expressed in muscle. These sequences are differentially spliced in patterns not reported for any striated muscle titin isoform. We used polyclonal antibodies raised against expressed protein fragments encoded by the Z-repeat region and the kinase domain region to investigate the relationship between the RT-PCR products and the c-titin protein. Both antibodies reacted with the c-titin band in Western blot analysis of platelet extracts and revealed immunofluorescent colocalization of at least some of the reactive protein with alpha-actinin, myosin, and actin in 3T3 cell stress fibers. These results suggest that differential expression of titin gene exons in nonmuscle cells yields multiple novel isoforms of the protein c-titin.

1087

**Cdk5 Modulates Pin1 Interactions with Beta and Delta-catenin Neuronal Cells**

J. P. Muñoz,[1,2] C. Huichalaf,[1,2] D. Orellana,[1,2] R. B. Maccioni[1,2]; [1]Cell Biology, Millennium Institute for Advanced Studies (CBB), Santiago, Chile, [2]Universidad de Chile, Santiago, Chile

The cdk5/p35 system has been implicated in a variety of functions related to brain development, including axonal outgrown and neuronal migration. In this study, on the basis of co-immunoprecipitation and pull-down experiments, we show that the cdk5/p35 complex associates with and phosphorylates the neuronal δ-catenin. Immunocytochemical studies of δ-catenin and the cdk5-activator p35 in primary cortical neurons indicated that these proteins co-localize in the cell body of neuronal cells. In addition, cdk5 co-localized with β-catenin in the cell-cell contacts and plasma membrane of undifferentiated and differentiated N2a cells. In this context, we identified $Ser_{191}$ and $Ser_{246}$ on β-catenin structure as the specific sites for the phosphorylation by the cdk5/p35 system. Moreover, Pin1, a peptidyl-prolyl isomerase (PPIase) directly bound to both β and δ-catenin once they have been phosphorylated by the cdk5/p35 complex. Studies indicates that the cdk5/p35 protein kinase system is directly involved in the regulatory mechanisms of neuronal β an δ catenin

1088

**Focal Contacts and the Formation of Keratin Filaments**

R. Windoffer, A. Koelsch, R. E. Leube; Anatomy and Cell Biology, Johannes Gutenberg University Mainz, Mainz, Germany

Given the spontaneous in vitro formation of intermediate filaments, complex regulatory mechanisms are needed in living cells for the adaptation of filament organization to specific spatial requirements. Thus, we observed formation of keratin filaments primarily in the cell periphery of epithelial cell lines during interphase, after mitosis and after disruption of the filament network using hyperphosphorylating drugs. By live cell microscopy we identified globular filament precursors arising in close vicinity of the cell edge. These precursors subsequently elongated, were transported retrogradely and finally fused either to each other or to an already existing network. In an attempt to identify structures that may be of importance for keratin filament precursor formation in the cell periphery we double transfected cell lines with fluorescent keratins and fluorescent focal contact proteins. Life cell imaging revealed a close spatial and temporal relationship between focal contacts and newly forming keratin filament precursors. Focal contacts may therefore act as regulators of keratin filament formation, possibly by changing the phosphorylation of soluble keratin assemblies and/or by providing structural scaffolds.

1089

**Constitutive Activation of Akt in Polyamine Depleted Cells Decreases Migration by Inhibiting GSK3beta and Rac1**

R. J. Vaidya, R. M. Ray, L. R. Johnson; Department of Physiology, University of Tennessee Health Science Center, Memphis, TN

The rapid migration of intestinal epithelial cells (IEC) is important for the healing of mucosal wounds. We have previously shown that polyamine depletion inhibits Rac1 and the migration of IEC-6 cells. Inhibition of Rac1 by expression of dominant negative Rac1, inhibition of Tiam1 or polyamine depletion caused the formation of thick actin cortex formation. Akt activation and its down stream target GSK3β have been implicated in the regulation of migration. In this study, we investigated whether elevated PI3-kinase/Akt signaling in response to polyamine-depletion decreases cell migration. Polyamine depleted cells had significantly higher PI3-kinase activity and Akt (Ser473/thr308) phosphorylation compared to controls. Pretreatment with 20 μM LY294002 (PI3-kinase inhibitor) inhibited phosphorylation of Akt, increased the migration, increased Rac1 activity in polyamine depleted IEC-6 cells, and restored the actin structure similar to that in cells grown in control medium. Inhibition of PI3-kinase reorganized the thick actin cortex into stress fibers. Treatment of cells with a GSK3β inhibitor altered actin cytoskeleton and inhibited migration, mimicking the effects of DFMO. Thus, our results indicate that sustained activation of Akt in the absence of polyamines inhibits migration through direct phosphorylation and inactivation of Rac1 or through GSK3β dependent signaling. (Supported by NIDDK grant DK-52784)

## Cytoskeleton-Membrane Interactions I (1090-1103)

1090

**Mechanotransduction in Fibroblasts is Mediated by the Na-H exchanger NHE1**

A. Shubov,[1] L. LeClaire,[1] N. Madson,[1] E. Almeida,[2] D. L. Barber[1]; [1]Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA, [2]NASA Ames Research Center, Moffett Field, CA

Mechanical forces regulate development, tissue growth and remodeling, and reparative responses. Our understanding of how cells sense and transduce mechanical force is limited compared with what we know about sensing and transducing chemical cues. In specialized sensory cells mechanotransduction units include a plasma membrane ion transport protein anchored to the actin cytoskeleton. We asked whether in fibroblasts an actin-anchored membrane ion transport protein functions in mechanotransduction. We found that the plasma membrane Na-H exchanger NHE1, which is anchored to actin filaments by binding ERM (ezrin, radixin, moesin) proteins, is mechanosensitive and functions in mechanotransduction in CCL39 fibroblasts. Cells plated on a fibronectin-coated polystyrene strip mounted in a uniaxial loading system were mechanically stimulated by applying 1% deformation. A 5 min mechanical load and elastic release induced a 2.5-fold increase in NHE1 activity and an NHE1-dependent increase in intracellular pH from 7.15 to 7.40. Mechanical loading also increased NHE1 phosphorylation, suggesting the action of a mechanosensitive kinase. Additionally, NHE1 was necessary for conserved responses to mechanical force. In CCL39 fibroblasts, mechanical loading increased activity of the focal adhesion kinase FAK 2.5-fold and recruitment of FAK to peripheral focal complexes. In NHE1-null PS120 cells derived from a parental CCL39 clone, FAK abundance and quiescent FAK activity were similar to that in CCL39 cells, but there was no increase in FAK activity or FAK recruitment with mechanical loading. The abundance of F-actin, although similar in unloaded CCL39 and PS120 cells, increased with mechanical loading of CCL39 cells ~2-fold, but was unchanged in PS120 cells. These data confirm that actin-anchored NHE1 is mechanosensitive and is necessary for transducing mechanoresponsive increases in FAK activity and F-actin assembly. Supported by NASA grant NNA04CC53A.

1091

**Labile and Dynamic Junctions of the Red Blood Cell Membrane Skeleton - A Potential Role for Tropomyosin**

M. A. Salomao,[1] X. An,[1] X. Guo,[1] A. J. Baines,[2] M. Narla[1]; [1]Red Cell Physiology Laboratory, New York Blood Center, New York, NY, [2]Department of Biosciences, University of Kent, Canterbury, United Kingdom

The ternary complex of spectrin-actin-4.1R is a central feature of erythrocyte membrane skeleton and it plays an important role in maintaining membrane stability. In the present study, we explored the dynamic regulation of this junctional complex in situ and the potential contribution of tropomyosin in stabilizing spectrin-actin-4.1R complex. We have taken advantage of an N-terminal fragment of β-spectrin (residues 1-301) that we have previously shown to inhibit formation of the spectrin-actin-4.1R ternary complex in vitro. Resealing this polypeptide into red cell ghosts prepared in the absence of magnesium chloride, which results in loss of tropomyosin, leads to a dose-dependent integration of the polypeptide into the pre-assembled spectrin-based membrane skeleton. The functional consequence of the integration of the polypeptide into the spectrin skeleton is a dose-dependent reduction in membrane mechanical stability. Furthermore, we show that when the ghosts were prepared in the presence of magnesium chloride, which maintains normal tropomyosin content of the membrane, the ability of spectrin polypeptide 1-301 to destabilize membrane skeleton is significantly reduced, suggesting spectrin-actin-4.1 complex is stabilized by tropomyosin. Importantly, reconstitution of erythrocyte tropomyosin into tropomyosin-depleted ghosts prior to addition of the 1-301 polypeptide once again resulted in reduced effectiveness of the peptide in decreasing membrane mechanical stability. By contrast, muscle tropomyosin did not protect membranes from destabilization by 1-301 peptide; evidently there is an isoform-specific requirement for tropomyosin in red cells. Based on these findings, we conclude that spectrin-actin-4.1R junctional complex is in a dynamic state in intact red blood cell membrane and that tropomyosin plays a key role in stabilizing spectrin-actin-4.1 complex.

1092

***Drosophila* Gap Junction Gene, *innexin-2*, is Required for Border Cell Migration**

S. Tazuke, M. T. Chan; CRRWH, University of Pennsylvania, Philadelphia, PA

OBJECTIVE: The mammalian gap junction gene, *connexin-43* (*cx43)*, is required for directed migration of cells such as neural crest cells during development. The specific mechanism of *cx43* mediated regulation of directed cell migration is unknown. We investigated the function of a gap junction gene in directed cell migration using Drosophila as a model organism, whose genome has reduced number of gap junction proteins, making genetic analysis far more tractable. METHOD: Genetic and phenotypic analysis of *innexin-2* (*inx-2*) loss of function in *Drosophila* ovaries. RESULTS: The migration of the border cells, a small group of follicle cells in the *Drosophila* ovary, provides a model system to study directed cell migration *in vivo*. We have found that *inx-2* gene product is expressed in the border cells. To investigate the function of *inx-2* , we transiently

expressed *hsp70-innexin2-EGFP* transgene during embryogenesis to rescue the embryonic lethality of *inx-2* null animals. We have found that loss of *inx-2* results in delayed border cell migration during stage 9-10 of *Drosophila* oogenesis. Notably, we found that *inx-2* function is required specifically for *par-6* expression and localization to the polarized apical membrane of the follicle epithelium. This is specific to *par-6* since other polarity proteins, *Drosophila* par-3, E-cadherin, *armadillo, atypical PKC* remain apically localized in *inx-2* null follicle cells. In mammalian cells, *Par-6* has recently been shown to regulate the disassembly of tight junctions during epithelial mesenchymal transition (Science. 2005 ;307:1603-9) . Our results suggest that *inx-2* mediated gap junctional intercellular communication play a role in cell migration by effecting *par-6,* which is required for the remodeling of cell-cell junctions during cell migration.

1093

**Effect of Colchicine on Acrosomal Biogenesis in Mouse Sperm**

W. Huang,[1] H. Ho[2]; [1]Life Science, National Taiwan University, Taipei, Taiwan Republic of China, [2]Anatomy, Tzu Chi University, Hualien, Taiwan Republic of China

The acrosome has a distinct shape and size depending on the species. The mechanisms regulating the attachment, shaping, and spreading of the acrosome over the nucleus is unclear. It is well known that microtubules play a key role in organelle and membrane-bound vesicle transportation in cells. Besides, spermatid nuclear shaping is dependent on manchette microtubules, it is possible that shaping and spreading of acrosome over the nucleus also depends on microtubules. In this study, colchicine, which binds to free tubulins and causes microtubule depolymerization, was used to evaluate the effects of microtubule disruption on acrosome formation in mouse spermatids. Control and colchicine-treated seminiferous tubules were investigated by thin sectioning, cytochemistry, and cryofracturing methods. In control cap phase spermatids, coated vesicles were always seen on the apex and caudal margins of the developing acrosome. The increase of volume and the accumulation of materials in the acrosome during Golgi and cap phases occurred via fusion of vesicles at various sites on the growing acrosome. By studying the acid phosphatase localization pattern and colchicine-treated spermatids, the role of clathrin-coated vesicles became clear. Coated vesicle formation at the caudal margin of the acrosome appeared to be responsible for spreading and shaping of the acrosome over the surface of the nucleus and also established distinct regional differences in the acrosome. In colchicine-treated spermatids, Golgi apparatus lost its typical membranous stack conformation and disintegrated into many small vesicles. Acrosome formation was retarded and there was discordance of spreading of the acrosomal cap with that of the modified nuclear envelope. Colchicine treatment indicated that microtubule-dependent vesicle trafficking between the Golgi apparatus and the acrosome plays vital role in acrosomal biogenesis. In addition, both anterograde and retrograde vesicle trafficking are extensively involved and equally important in acrosome formation.

1094

**Sun1, an Inner Nuclear Membrane Protein, Mediates the Anchorage of Nesprin-2 to the Nuclear Envelope**

W. Lu; Institute for Biochemistry, Koeln, Germany

Nesprins form a novel class of nuclear envelope anchored spectrin repeat proteins. We show that a direct association of their highly conserved C-terminal luminal domain with the inner nuclear membrane protein Sun1 mediates their nuclear envelope localization. In Nesprin-1 and Nesprin-2 the conserved C-terminal amino acids PPPX are essential for the interaction with a C-terminal region in Sun1. In fact, Sun1 is required for the proper nuclear envelope localization of Nesprin-2 as shown with dominant negative mutants and by knockdown of Sun1 expression. Sun1 itself does not require functional A-type lamins for its localization at the inner nuclear membrane in mammalian cells. our findings propose a conserved nuclear anchorage mechanism between *C. elegans* and mammals and suggest a model in which Sun1 serves as a “structural bridge” connecting the nuclear interior with the actin cytoskeleton.

1095

**Cytoskeletal Organization Surrounding the Nucleus Mediated by Bpag1**

K. Young, R. Kothary; Ottawa Health Research Institute, Ottawa, ON, Canada

Bullous pemphigoid antigen 1 (Bpag1) proteins are large cytoskeletal organizing proteins. They are critical for normal architecture in the skin, muscle and nervous system, with neuromuscular dysfunction and early postnatal death occurring in Bpag1 mutant (dystonia musculorum - dt) mice. One of the characteristic abnormalities observed early on in neurons from dt mice was eccentricity of the nuclei. In this study, we have characterized an isoform of Bpag1 (Bpag1 isoform 2) which contains a novel N-terminal transmembrane domain and associates with membranous structures surrounding the nucleus. Bpag1 isoform 2 primarily associated with actin filaments, and accumulated F-actin around the nucleus and Golgi apparatus in Cos-1 and C2C12 cells. This indicates a role in nuclear tethering and structural organization around the nucleus. Bpag1 isoform 2 can also enter into the nucleus, and may mediate structure within the intranuclear space. Comparisons of Bpag1 isoform 2 are made to the isoform 1 variant and distinct features are noted. The overall structure and localization of Bpag1 isoform 2 indicates that it is a nesprin-like protein, with possible similar or overlapping roles.

1096

**Distinctive Distribution of Protein 41 Homologs in *Mouse* Adrenal Gland**

C. Liu, X. An, Y. Wu, G. Debnath, M. Narla; Red Cell Physiology, New York Blood Center, New York, NY

Protein 4.1 is an adapter protein that links the actin-based cytoskeleton to various transmembrane proteins. The family of 4.1 proteins is encoded by four homologous genes, 4.1R, 4.1G, 4.1N, and 4.1B, which undergo complex alternative splicing. Both in situ hybridization and Northern blot analysis show that various protein 4.1 homologs are expressed in adrenal gland. However, the cellular and sub-cellular localization of these proteins in the adrenal gland has not been defined. As a first step in defining the function of protein 4.1 homologs in adrenal gland, we systemically examined the distribution and localization of these proteins in the gland by staining formaldehyde-fixed paraffin-embedded mouse adrenal gland sections with antibodies highly specific for each of the four 4.1 protein homologs. We find that all four 4.1 protein homologs are expressed in all adenocortical and adenomedulla zones but not in zona reticularis. However, we noted distinct subcellular localization of the various homologs. While 4.1R is restricted to nuclei, 4.1N accumultaed in peri-nuclear region. 4.1G is diffusely distributed in the cytoplasm while 4.1B exhibited both cytoplasmic and membrane localization. Furthermore, immuno-gold electron microscopy showed that 4.1B is present in the granules of chromaffin

cells of adrenal medulla. The diverse cellular distribution and distinct expression pattern of 4.1 proteins in mouse adrenal gland indicates diverse roles for 4.1 proteins in endocrine functions of adrenal gland.

1097

### Morphological Analysis of Native Membrane Cytoskeleton under Cryo-electron Microscope

J. Usukura,[1] M. Makihara,[2] T. Watanabe,[3] N. Arimura,[3] N. Tanaka,[2] K. Kaibuchi[3]; [1]Anatomy and Cell Biology, Nagoya University, Nagoya, Japan, [2]Applied Physics, Nagoya University, Nagoya, Japan, [3]Cell Pharmacology, Nagoya University, Nagoya, Japan

**Purpose:** This study aims to elucidate molecular interaction between membrane and cytoskeleton by *in situ* morphological analysis. Now we established a new method to observe directly hydrated membrane cytoskeleton in native state under cryo-electron microscope while identifying constituent molecules by immuno-labeling. **Methods:** The grid meshes covered with carbon coated Formval film and treated subsequently with 1% Alucian blue were employed for sticking the cell membrane. Apical cell membranes stuck on the gird by contacting such grids on the cell surfaces were immediately frozen by plunging it into liquid ethane for morphological observation. For identification of protein molecules, membrane stuck on the grid was fixed briefly with 1 % glutaraldehyde and incubated with primary and subsequent secondary antibodies prior to quick freezing as described elsewhere. **Results and Discussion:** Even in hydrated and frozen state without any treatments, actin filaments, microtubules, clathrin coat and ER were clearly identified within 5 to 10 micron defocus. In particular, microtubules just beneath the membrane appeared to be abundant and flexible or curved. The average thickness of actin filaments, microtubules and clathrin coat in hydrated state were 7.6nm, 25.1nm and 6.8nm respectively. On considering regulation mechanism of morphogenesis, IQGAP1, an effector of Rac1 and actin-binding protein, accumulates at lamellipodia. Indeed, IQGAP1 was localized more dominantly on actin filaments closely attached to the cell membrane, but not on actin stress fibers. Those results were well compatible with the observation by imnuno-replica method. **Conclusion:** New methods consisting of membrane acquisition and cryo-techniques was available for structural analysis of membrane cytoskeleton in hydrated native state.

1098

### Proteomic Analysis of the Membrane Skeleton of *Tetrahymena thermophila*

P. C. Anderson,[1] A. L. Wentland,[1] D. J. Beussman,[1] R. B. Fulton,[2] M. Murakami,[2] E. S. Cole,[2] H. Said,[3] J. E. Honts[3]; [1]Chemistry, St. Olaf College, Northfield, MN, [2]Biology, St. Olaf College, Northfield, MN, [3]Biology, Drake University, Des Moines, IA

The membrane skeleton of the ciliated protozoan *Tetrahymena* contributes to the shape and cortical organization of this complex eukaryotic cell. The availability of genomic sequence data for *Tetrahymena thermophila* has facilitated a proteomic analysis of the non-microtubular portion of this cell's cortical cytoskeleton. Shape-preserving cortical residues, depleted of microtubules, can be prepared by extraction of *Tetrahymena* cells with potassium iodide in the presence of Triton X-100. The membrane skeletal residues obtained by this treatment were fractionated into distinct sets of proteins by selective extraction and precipitation procedures. Bands containing the constituent proteins were excised from preparative SDS-polyacrylamide gels. These proteins were reduced, alkylated and trypsin digested in-gel and the resulting enzymatic peptide fragments analyzed by LC-MS using electrospray ionization. The observed peptide molecular weights were used to search a database of predicted *Tetrahymena* proteins in order to identify the protein in the original gel band. Further confirmation was obtained by sequencing several enzymatically-derived peptides in the mass spectrometer. Two major components were identified - epiplasmin C and epiplasmin A - both of which are predicted to be coiled coil proteins. A related protein, epiplasmin B, was also detected. The other major protein identified was TCBP-25, an EF-hand calcium-binding protein. Intriguingly, among minor components present in these membrane skeletal residues were proteins related to protein and lipid kinases, as well as another EF-hand containing protein. These minor constituents suggest the possibility of a linkage between the structural cytoskeletal proteins that comprise the membrane skeleton and regulatory proteins involved in cellular signaling processes.

1099

### A Novel Role of Calpain in Membrane Ruffling Involves Deregulation of Cofilin 1, Tropomyosin 1 and RhoGDI-1

A. K. Larsen,[1] R. Lametsch,[1] T. B. Farre,[1] J. S. Elce,[2] M. Larsen,[3] B. Thomsen,[4] P. Ertbjerg,[1] M. A. Lawson[1]; [1]Department of Dairy and Food Science, Royal Veterinary and Agricultural University, Frederiksberg C, Denmark, [2]Department of Biochemistry, Queen's University, Kingston, ON, Canada, [3]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark, [4]Department of Genetics and Biotechnology, Danish Institute of Agricultural Sciences, Tjele, Denmark

Accurate regulation of the actin cytoskeleton is essential for a number cell motility processes including migration, spreading and the formation of lamellipodia, filopodia and membrane ruffles. The proteases µ- and m-calpain are involved in integrin-mediated cytoskeletal rearrangements, required for cell migration and cell spreading, by their modulation of focal adhesion components and signalling molecules. Here, we provide evidence that calpains are also essential in membrane ruffling. Calpain-deficient (*capn4*$^{-/-}$) fibroblasts ruffled significantly less than wild type (*capn4*$^{+/+}$) fibroblasts, in live cells grown on uncoated glass-surfaces. Active, GFP-tagged m-calpain was expressed in *capn4*$^{-/-}$ fibroblasts and fully restored the ruffling phenotype, as determined by time-lapse fluorescence microscopy. As shown previously, expression and activity of m-calpain-GFP in *capn4*$^{-/-}$ fibroblasts required co-expression of exogenous calpain small subunit. The fusion protein localized diffusely in the cytoplasm of *capn4*$^{-/-}$ fibroblast both under un-stimulated and $Ca^{2+}$-stimulated conditions, indicating that no major translocation of m-calpain to cell membranes occurred. To asses which proteins might influence the ruffling phenotype, proteome analysis of the two cell lines was carried out. Interestingly, a markedly increase in the level of tropomyosin 1, and decreased levels of cofilin 1 and RhoGDI-1 was detected in *capn4*$^{-/-}$ cytoplasmic lysates, compared with the wild type cells. Cofilin and tropomyosins are key regulators of actin filament dymamics, whereas RhoGDIs regulates activities of Rho-family GTPases. Together these data suggest that deregulation of these proteins is linked with the observed differences in ruffling, mediated by calpain.

1100

### Integrins and Platelet Glycoprotein Ibα Interact with Distinct Filamin A Domains and Crystal Structures Reveal that They Have Similar Binding Interfaces

T. Kiema,[1] R. Pudas,[1] Y. Lad,[2] F. Nakamura,[3] P. Jiang,[4] K. L. Wegener,[4] I. D. Campbell,[4] O. Heikkinen,[5] P. Permi,[6] I. KIlpeläinen,[5,6] J. H. Hartwig,[7,8] T. P. Stossel,[7,8] D. A. Calderwood,[2] J. Ylanne[1,9]; [1]Department of Biochemistry, University of Oulu, Oulu, Finland, [2]Department of

Pharmacology, Yale University, New Haven, CT, [3]Hematology Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA, [4]Department of Biochemistry, University of Oxford, Oxford, United Kingdom, [5]Department of Chemistry, University of Helsinki, Helsinki, Finland, [6]Institute of Biotechnology, University of Helsinki, Helsinki, Finland, [7]Hematology Division, Brigham and Women's Hospital, Boston, MA, [8]Medical School, Harvard University, Boston, MA, [9]Department of Biological and Environmental Sciences, University of Jyvaskyla, Jyvaskyla, Finland

Cytoskeletal association with transmembrane proteins permits transmission of biochemical signals and mechanical force across the plasma membrane and is essential to the development and functioning of multicellular animals. Here we report the first atomic level characterization of interactions of filamin A with the cytoplasmic face of transmembrane receptors, integrin and glycoprotein Ibα (GPIbα). The binding studies showed that a major interaction site with the cytoplasmic tail of integrin resides at the filamin A immunloglobulin-like domain 21 (FLNa21) and that the GPIbα binds to the filamin A domain 17 (FLNa17). The crystal structure of the FLNa21 with the integrin β7 cytoplasmic tail peptide showed that the integrin peptide forms an additional β-strand next to the β-strand C of the FLNa21 and that several hydrophobic residues of the integrin are involved in the interaction. Mutation and NMR studies confirm that the interaction seen in the crystal also takes place in solution and in cells. The complex structure of the FLNa17 with the GPIbα peptide showed a similar extension of the β-sheet and similar hydrophopic contacts as were observed for integrin-filamin interaction, but also some specific hydrophopic contacts were seen. Point mutations in the FLNa17 abrogated full-length filamin A binding to GPIbα in co-immunprecipitation studies. The striking similarity of the interaction of integrin and GPIbα peptides with the filamin domains seen in the crystal structures suggests that it is a general mode of ligand binding by immunoglobulin-like filamin repeats and could enlighten the analysis of the many other reported filamin-ligand interactions. Furthermore, the structures suggest mechanisms how integrin-FLNa interaction could be regulated by phosphorylation or by competition with talin, and why sulfhydryl modifications abrogate the GPIbα filamin interaction.

1101

## Pecam-1 Cytosolic Domain Regulates Endocytosis and Actin Reorganization Triggered by Pecam-1 Clustering

A. Thomas,[1] C. Garnacho,[1] S. Muro,[1,2] S. Albelda,[3] M. Koval,[4] V. Muzykantov[1,2]; [1]Institute for Environmental Medicine, University of Pennsylvania Medical School, Philadelphia, PA, [2]Department of Pharmacology, University of Pennsylvania Medical School, Philadelphia, PA, [3]Division of Pulmonary, Allergy and Critical Care Medicine, University of Pennsylvania Medical School, Philadelphia, PA, [4]Division of Pulmonary, Allergy and Critical Care Medicine, Emory University, Atlanta, GA

Platelet-Endothelial Cell Adhesion Molecule (PECAM)-1 is an Ig-superfamily transmembrane glycoprotein involved in endothelial layer integrity, angiogenesis and leukocyte transmigration. We found that nanocarriers carrying PECAM antibodies (anti-PECAM/NCs) enable intracellular drug delivery into endothelium via CAM-mediated endocytosis, induced by PECAM-1 clustering. These findings suggest dynamic signaling from PECAM-1 to the actin cytoskeleton. Using fluorescence microscopy, we explored the role of the PECAM cytosolic domain in actin reorganization required for CAM-mediated endocytosis of FITC-labeled anti-PECAM/NCs. We used REN cells transfected with either full length PECAM containing cytosolic exons 10-16, PECAM mutants with the cytosolic tail truncations Δ10-16, Δ11-16, Δ15-16, or mutated at two candidate signaling residues (Y663F in exon 13 or Y686F in exon 14). Endocytosis of anti-PECAM/NCs and actin reorganization into stress fibers were observed in REN cells expressing full-length PECAM-1, as well as Δ15-16 and Y663F mutants. However, both processes were inhibited in cells expressing Δ10-16, Δ11-16, and Y686F PECAM-1 mutants. Activated Rac was detected in REN cells expressing full-length PECAM-1, which was enhanced upon binding of anti-PECAM/NCs to the cells, while activated Rac was undetectable in Δ10-16 PECAM-1 mutant. In addition, anti-PECAM/NCs induced Rho activation in REN-PECAM-1 cells, but this was attenuated by Δ10-16 truncation. These data indicate that PECAM-1 cytosolic domain regulates actin cytoskeleton in response to extracellular ligands (anti-PECAM/NCs), which is likely mediated by PECAM-1 tyrosine residue Y686, and operates through Rac and Rho. Actin-mediated changes in endothelial cell shape, which contribute to transmigration (induced by PECAM-1-adhered leukocytes), or angiogenesis (induced by integrin-mediated PECAM-1 de-phosphorylation) may operate by a similar pathway.

1102

## Comparative Studies of Erythroid Spectrin and Non-erythroid Spectrin Repeats

X. An, X. Guo, M. Salomao, X. Zhang, Y. Wu, A. Baines, N. Mohandas; Red Cell Physiology Laboratory, The New York Blood Center, New York, NY

Spectrins are long rod-like, flexible molecules with multiple functions. Spectrins are composed of α- and β-spectrin subunits which form anti-parallel dimers through lateral association, and tetramers through head-to-head association of dimers. Two α-subunits (αI, αII) and five β-subunits (βI-V) have been identified in humans. The structure and function of erythroid spectrin (αIβI) have been well studied, while the structure and function of other spectrins is less well defined. In the present study, we performed a series of analyses to compare αIβI with αIIβII-spectrin, an isotype abundant in most non-erythroid tissues. We compared the conformational stability of individual triple-helical repeats, the ability of individual repeats to bind phosphatidylserine and their dimerization characterisitics. Our findings are as follows. (1) αIIβII single repeats are more conformationally stable than their counterparts in erythroid spectrin. (2) During temperature induced unfolding of tandem repeats, αIIβII repeats unfold more cooperatively than those of erythroid spectrin. (3) Of the 10 phosphatidylserine binding repeats in the erythroid spectrin chains, 5 are conserved in αIIβII; one cluster of 3 consecutive structural repeating units in αI-spectrin (repeats 8-10) is displaced by one repeat in αII-spectrin (repeats 9-11). Repeat 1 of αII-spectrin binds phosphatidylserine, unlike the corresponding repeat of αI-spectrin. (4) The interaction of α and β polypeptides to form dimers requires β repeats 1-2 and α repeats 20-21 in all cases. However, the interaction between αII-βII dimerization peptides appears to be significantly stronger than that of erythroid spectrin. Our data suggests that αI/αII and βI/βII polypeptides have been subfunctionalized in evolution. αIβI is evidently adapted to the flexible, elastic nature of erythrocyte membranes; αIIβII is adapted to provide stability to membrane structures in complex tissues.

1103

**The Blood Group Antigen-carrying Protein XK is Linked to Red Cell Membrane Skeleton Through its Interactions with Protein 41R and Alpha Spectrin**

X. Zhang, X. An, X. Guo, Y. Wu, M. Narla; Red Cell Physiology, New York Blood Center, New York, NY

The red blood cell membrane is a composite structure in which spectrin-actin based membrane skeletal network is linked to lipid bilayer. The attachment of membrane skeleton to lipid bilayer is mediated either by direct interaction of skeletal protein with lipids (such as the interaction of spectrin and 4.1R to phosphatidylserine) or by interaction of skeletal proteins to cytoplasmic domains of transmembrane proteins. To date, two such vertical protein linkages have been well studied. One is band 3-ankyrin-spectrin interaction and the other is Glycophorin C-protein 4.1R interaction. Recent evidence indicates that many other transmembrane proteins such as XK, Kell, Lu, Rh/RhAG are also linked to membrane skeleton. However, much less is known regarding molecular basis for these interactions. In the present study, we show that the expression of XK is significantly reduced in red blood cells of 4.1R knockout mice: this is analogous to an earlier finding that glycophorin C content of these membranes is markedly reduced, and strongly implies that XK interacts with 4.1R in situ. In vitro pull down assay demonstrated that the cytoplasmic domain of XK binds to the N-terminal 30 kDa membrane-binding domain of 4.1R. Importantly, the interaction of XK with protein 4.1R is regulated by $PIP_2$. We also show that the cytoplasmic domain of XK binds to alpha spectrin; the binding site on spectrin is located within the EF hands at the C-terminus of alpha spectrin. These findings enable us to identify new protein linkages in the red cell membrane and thus further our understanding of its structural organization.

## Cell Attachment to the Extracellular Matrix (1104-1124)

1104

**Bifunctional Peptides Derived from Homologous Loop Regions in the Laminin α Chain LG4 Modules Interact with Both α2β1 Integrin and Syndecan-2**

F. Yokoyama,[1,2] N. Suzuki,[1] Y. Kadoya,[3] Y. Kikkawa,[2] M. Haruki,[1] H. K. Kleinman,[4] M. Nomizu[2]; [1]Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan, [2]School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan, [3]Department of Anatomy, Kitasato University School of Medicine, Sagamihara, Japan, [4]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD

Laminin α chains show diverse biological functions in a chain-specific fashion. The laminin G-like modules (LG modules) of the laminin α chains consist of a 14-stranded β-sheet sandwich structure with biologically active sequences found in the connecting loops. Previously, we reported that connecting loop regions between β-strands E and F in the mouse laminin α chain LG4 modules showed chain-specific activities. In this study, we focus on the homologous loop regions in human laminin α chain LG4 modules using five synthetic peptides (hEF-1-hEF-5). These homologous peptides induced chain-specific cellular responses in various cell types. Next, in order to examine dual-receptor recognition model, we synthesized chimeras (cEF13A-E) derived from peptides hEF-1 and hEF-3. All of the chimeric peptides promoted fibroblast attachment as well as the parental peptides. Fibroblast attachment to cEF13A and cEF13B was inhibited by anti-integrin α2 and β1 antibodies and by heparin, while cell adhesion to cEF13C, cEF13D, and cEF13E was blocked only by heparin. Actin organization of fibroblasts on cEF13C was not different from that on hEF-3, but cEF13B induced membrane ruffling at the tips of the actin stress fibers. These results suggest that cEF13B had bifunctional effects on cellular behaviors through α2β1 integrin and heparin/heparan sulfate proteoglycan. We conclude that the approach utilizing chimeric peptides is useful to examine cellular mechanisms in dual receptor systems.

1105

**Characterization of α-Dystroglycan Binding Sequence Derived from the Laminin α2 Chain LG4-5 Module**

T. Yoshimura,[1] N. Suzuki,[1,2] F. Yokoyama,[1,2] Y. Kikkawa,[1] J. Yamada,[1] M. Nomizu[1]; [1]School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan, [2]Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan

Laminins are major components of basement membranes and have various biological activities. Laminins consist of α, β, and γ chains. The C-terminal globular domain of the α chain (G domain) consists of five LG modules (LG1-5) and plays a critical role for the biological activity. The laminin α2 chain, which is a component of laminin-2/4/12, is mainly expressed in skeletal muscles or peripheral nerves and interacts with dystroglycan and heparan sulfate proteoglycans. Here, we focused on the laminin α2 chain LG4-5 module and to identify their heparin and dystroglycan binding activities to better understand the biological function of the molecule. We prepared a recombinant laminin α2 chain LG4-5 module (rec-α2LG4-5) using 293T cells. The rec-α2LG4-5 showed heparin and α-dystroglycan binding activities. To identify the binding sites in the laminin α2 chain LG4-5 module, we synthesized 42 overlapping peptides covering the entire molecule. First, we examined the effect of the peptides on the heparin binding of rec-α2LG4-5. The A2G78 peptide (GLLFYMARINHA, mouse laminin α2 chain 2796-2807) inhibited the heparin binding of rec-α2LG4-5. A2G78 showed heparin binding activity in a solid phase binding assay. The minimal active sequence of A2G78 for heparin binding was found to be LLFYMARI. Next, we tested the α-dystroglycan binding activity of the A2G78 sequence. The A2G78 peptide showed inhibitory effect on α-dystroglycan binding to the rec-α2LG4-5 protein. These results suggest that the A2G78 site plays an important role in the interaction of rec-α2LG4-5 with heparin/heparan sulfate and α-dystroglycan.

1106

**A Role for $\alpha_4\beta_1$ Integrin-Specific Fibronectin Peptides in Enhancing Small Intestinal Submucosa (SIS) Vascular Graft Biocompatibility**

E. J. Rodenberg,[1] M. C. Hiles,[2] F. M. Pavalko[1]; [1]Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN, [2]Cook Biotech, Inc., West Lafayette, IN

The limited availability of autologous vessel transplant grafts and high failure rates of current-day synthetic graft prostheses create the need to engineer a more compatible, implantable vascular biomaterial. We propose using acellular, Type I collagen-enriched porcine small intestinal submucosa (SIS) as an ideal biomaterial. Previously, we demonstrated that human endothelial cells cultured on SIS “condition” the surface and render it more biocompatible to subsequent endothelial cell adhesion through the secretion of a fibronectin-rich extracellular matrix. However, platelets express integrins which recognize and bind the Arg-Gly-Asp-Ser (RGDS) tetrapeptide within intact fibronectin and collagen. Others have

shown the $\alpha_4\beta_1$ integrin receptor recognizes two specific peptide sequences, Leu-Asp-Val (LDV) and Arg-Glu-Asp-Val (REDV), within human plasma fibronectin (Komoriya *et al*., Mould *et al*.). We hypothesize that crosslinking SIS with non-platelet specific fibronectin binding peptides can increase endothelial adhesion and decrease platelet binding. To test this hypothesis, we first wanted to determine if platelets, endothelial cells, and human fibroblasts express $\alpha_4\beta_1$. Immunoprecipitation and Western blot analyses demonstrated surface expression of $\alpha_4$ and $\beta_1$ integrin subunits on human umbilical vein endothelial cells (HUVEC), human coronary artery endothelial cells (HCAEC), and human foreskin fibroblasts (HFF). Fluorescence-activated cell sorting (FACS) analysis confirmed that neither non-activated nor thrombin-activated human platelets express the $\alpha_4$ integrin subunit. Currently, we are investigating the ability of HUVEC, HCAEC, HFF, and platelets to recognize and bind to fibronectin peptide fragments, including REDV, RGDS, and LDV, immobilized on 96-well microplates. To quantitate total binding, immunofluorescence labeling and Image Tool® software analysis are being used. In conclusion, cross-linking $\alpha_4\beta_1$ integrin-specific peptides to SIS may yield a more compatible vascular biomaterial that would allow for endothelial attachment while significantly minimizing platelet attachment. Funding: Cook Biotech, Inc. and NIH R01 AR-049728 (FMP).

1107

**Determination of the Critical Amino Acid Residues within the EIIIA (ED-A) Segment of Fibronectin that Bind Integrins α9β1 and α4β1**

A. V. Shinde,[1] C. Bystroff,[2] C. Wang,[2] P. Vincent,[3] L. Van De Water[1]; [1]Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY, [2]Department of Biology, Rensselaer Polytechnic Institute, Troy, NY, [3]Center for Cardiovascular Sciences, Albany Medical College, Albany, NY

Fibronectins (FNs) are a family of extracellular glycoproteins that mediate cellular adhesion, migration and differentiation. The FNs are disulfide-bonded, dimeric proteins with each chain composed of homologous repeats of three prototypical types, termed FN-I, FN-II, FN-III. Each FN monomer contains a contiguous array of 15-17 of the ~90 amino acid FN III repeats. Increased expression of cellular FNs that contain the alternatively spliced EIIIA and/or EIIIB FN III segments is a prominent feature during embryogenesis, wound healing, tumor progression and inflammation. We previously reported that two integrins, α9β1 and α4β1, ligate the EIIIA segment (Liao et al J. Biol. Chem. 2002) and that the epitopes for function-blocking monoclonal antibodies lie within the C-C' loop of EIIIA (Liao et al J. Biol. Chem. 1999). We have now created a library of point mutants within rat EIIIA and performed cell adhesion assay on these single and double mutants. We find that the Asp41 and Gly42 within the C-C' loop are critical in mediating integrin binding. We designed synthetic peptides based on the predicted important amino acid sequence from the C-C' loop, characterised their structure by NMR and identified peptides that inhibit α9β1 and α4β1-mediated cell adhesion, using SW480 α9-transfected cells and α4β1-expressing HFF-2 and HFL-1 cells. Our data provide an important basis for understanding the functions of the alternatively spliced FNs in normal and pathogenic settings.

1108

**The Structures of the Thrombospondin-1 N-terminal Domain and its Complex with a Synthetic Pentameric Heparin**

K. Tan,[1] M. Duquette,[2] J. Liu,[1] J. Wang,[1] J. Lawler[2]; [1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, [2]Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA

The N-terminal domain of thrombospondin-1 (TSPN-1) mediates the protein's interaction with (1) glycosaminoglycans, calreticulin and integrins during cellular adhesion and migration, (2) low-density lipoprotein receptor-related protein (LRP) during uptake and clearance and (3) fibrinogen during platelet aggregation. The structure of TSPN-1, as determined by X-ray crystallography to 1.8 Å resolution, is a b-sandwich with 13 anti-parallel b strands and one irregular strand-like motif comprising a concaved front sheet and a convex back sheet. Topologically, it belongs to the concanavalin A-like lectins/glucanases superfamily. However, its unique structural features at the N- and C-terminal regions, and the disulfide bond location distinguish TSPN-1 from other members of this superfamily. Binding sites for calreticulin, fibrinogen and a4b1 integrin that have been identified using synthetic peptides map to the various edges of the domain and appear to be available for binding to these ligands. The crystal structure of the complex of TSPN-1 and a pentameric heparin indicates that residues R29, R42 and R77 in an extensive positively charged patch at the bottom of the domain specifically associate with the sulfate groups of heparin. The TSPN-1 structure also identifies a flexible linker between the globular TSPN-1 domain (N1-C214) and the coiled-coil sequence region that serves as the trimerization site. The TSPN domain is found in various extracellular matrix molecules including TSPs, NELLs, many collagens, TSPEAR and kielin. Whereas the sequence identity of the TSPN domains is relatively low for these proteins, the hydrophobicity pattern of key positions that define the b strands of TSPN-1 and the positions of the two cysteine residues that form a disulfide bond are similar in all of them. These data indicate that the TSPN domain represents a distinct subclass of the concanavalin A-like lectins/glucanases superfamily.

1109

**KGF Gene Transfected Colorectal Cancer Cells Induced Cell Adhesion to Extracellular Matrix**

M. Kudo, T. Ishiwata, N. Nakazawa, K. Kawahara, T. Fujii, Y. Kawamoto, K. Teduka, T. Suzuki, Z. Naito; Department of Integrative Pathology, Nippon Medical School, Tokyo, Japan

It has been reported that, in the case of colorectal cancer, keratinocyte growth factor (KGF) was expressed in neuroendocrine cells which were found to be both in close proximity to tumor cells, and also in tumor cells and as well as in the adjacent stromal fibroblasts. KGF is suggested to act either in an_autocrine or in paracrine manner to regulate colorectal cancer cell functions and cell behaviors. In the present study, we examined the role of KGF in adhesion activities of colorectal cancer cells by using a KGF gene transfected- and stably expressing-cell line HCT-15KGF that had been constructed from a colorectal cancer cell line, HCT-15. The original HCT-15 expresses KGFR and a very low level of KGF. HCT-15KGF cells also express KGFR and secrete 2.0 ± 0.06 ng/ml of KGF in the culture medium. Expression levels of integrin mRNAs such as β-1, α-5 and α-v that were relevant to the adhesion of cells to fibronectin showed no significant difference between HCT-15KGF and mock-transfected HCT15-MOCK cells. HCT-15KGF cells showed markedly increased adhesiveness to ECM; such as fibronectin, laminin and type IV-collagen. Activity of a mitogen-activated protein kinase (MAPK), ERK1/2, was increased in HCT-15KGF compared with HCT-15MOCK cells. The adhesion of the cells to fibronectin was inhibited by the treatment of the cells with ERK1/2 inhibitor, U0126, implying that a MAPK pathway is involved in adhesiveness of the cells to fibronectin. The data indicates that over-expression of KGF in colorectal cancer cells induces the cell adhesion to ECM through the MAPK pathway.

1110

**AFM Measurements of Forces Linking Hyaluronic Acid to CD44 Show Change in its Cytoskeletal Association in Glioma U-373**

C. Lamontagne-Carpin, M. Grandbois; Department of Pharmacology, Université de Sherbrooke, Sherbrooke, PQ, Canada

Glioblastomas are invasive cells that infiltrate very aggressively brain tissues and surgical interventions have therefore little chances of success. Hyaluronic acid (HA), a linear high molecular weight glycosaminoglycan, is a major component of the extra-cellular matrix in the brain and has been shown to be involved in cellular migration of highly invasive gliomas through is interaction with the cell receptor CD44. However the regulation of cell avidity for this polysaccharide is controlled by a large number of molecular mechanisms, which are still little understood. In the so-called force spectroscopy mode, the atomic force microscope (AFM) permits mechanical characterization of single bimolecular events. Here, HA / CD44 interactions were measured by allowing an AFM tip functionalized with HA molecules to contact the surface of glioblastomas U-373 expressing the CD44 receptor. The unbinding forces, when reported on a histogram, clearly exhibit two populations. First, in the low force regime (15 pN), we can appreciate the disrupt ion of HA / CD44 interaction. A second population (25 pN) with force elongation profile characteristic of membrane tether pulled from the cell surface can be observed. This finding is supported by the extent of the high force population (membrane tethers) that can be modulated through the interaction of the cytoskeleton with the cell membrane. Hence, cytoskeleton disruption by latrunculin, increase the high force regime population whereas the PMA treatment, which has shown to reorganize the actin network increasing CD44 / cytoskeleton interactions, leads to ruptures corresponding to the low force regime. As a conclusion, it is likely that both interactions will have a deep influence on the cell behavior toward HA, therefore foreseeing interesting principles in gliomas invasiveness.

1111

**Adsorption of Distinct ECM Proteins and Subsequent Remodeling Control the Osteogenic Differentiation of Mesenchymal Stem Cells on Polymeric Scaffolds**

A. K. Kundu, A. J. Putnam; Chemical Engineering and Materials Science; Biomedical Engineering, University of California, Irvine, Irvine, CA

Several recent studies highlight the use of multipotent adult bone marrow-derived mesenchymal stem cells (MSCs) for tissue engineering applications. Predictably controlling MSC differentiation is vital to widen their clinical use. While the roles of various soluble factors in promoting osteogenic differentiation have been studied, very little is known about the effect of the extracellular matrix (ECM) on MSC differentiation. In the present study, we compared the osteogenic potential of MSCs cultured on thin films of poly(lactide-co-glycolide) (PLGA) or poly(caprolactone) (PCL), two polymeric biomaterials widely used in tissue engineering as synthetic ECM analogs and that have been previously reported to differentially support osteogenic differentiation. We hypothesized that the adsorption of distinct ECM proteins present in serum-containing culture medium and subsequent differences in integrin-mediated adhesion account for the differential abilities of these materials to support osteogenesis. Supporting this hypothesis, significant amounts of fibronectin and vitronectin deposited onto both materials in serum-containing osteogenic media, with type-I collagen present in lower amounts. Adhesion-blocking studies revealed that MSCs adhere to PCL primarily via vitronectin, while type-I collagen mediates their attachment to PLGA. Moreover, de novo synthesis of ECM proteins by the MSCs occurred after prolonged periods of culture, suggesting that ECM remodeling plays an important role in the osteogenic process in vitro. These distinctions in the initial adhesive mechanisms and subsequent cell-mediated remodeling of the ECM correlated with higher levels of alkaline phosphatase activity and osteopontin expression after 2 weeks of monolayer culture in MSCs grown on PLGA rather than PCL. Together, these data suggest the initial integrin-mediated adhesion and subsequent matrix remodeling are critical regulators of osteogenesis, and stress the need for an improved molecular understanding of cell-ECM interactions in stem cell-based therapies.

1112

**Directed Cell Growth on Protein Functionalized Hydrogel Surfaces**

M. R. Hynd, J. Frampton, N. Dowell-Mesfin, J. N. Turner, W. Shain; Department of Health, Wadsworth Center, Albany, NY

Biochemical surface modification has been used to direct cell attachment and growth on a biocompatible gel surface. The spatial patterning of cells was based on the microcontact printing of protein molecules onto a gel surface. Acrylamide-based hydrogels were photopolymerized in the presence of an acroyl-streptavidin monomer to create planar, functionalized surfaces capable of binding biotin-labeled proteins. Microcontact printing was used to transfer the biotinylated extracellular matrix proteins, fibronectin and laminin, and the laminin peptide, IKVAV, onto modified surfaces. Proteins were patterned using PDMS stamps possessing a variety of surface designs. As a biological assay, LRM55 astroglioma cells and primary hippocampal neurons were plated on stamped hydrogels. Fluorescence, light and confocal microscopy were used to quantify cell attachment and cell morphology on modified surfaces. Cells were found to selectively adhere to areas stamped with biotin-conjugated proteins only. No cell attachment was observed within the un-patterned areas. Cells remained attached to the patterned areas for >4 weeks. Results from this study suggest that hydrogel surfaces can be patterned with multiple proteins to direct cell growth and attachment. This technique may be used to modify nanofabricated neural prosthetic devices to enhance biocompatibility. *This work was supported in part by the Nanobiotechnology Center (NBTC), an STC Program of the National Science Foundation under Agreement No. ECS-9876771.*

1113

**Crosslinked Hyaluronan with PEG Nanostents Inhibits Collagen Gel Contraction: A Potential Preventative of Burn Contracture**

K. Ghosh,[1] T. Mehra,[1] X. Z. Shu,[2] G. D. Prestwich,[2] R. A. F. Clark[3]; [1]Biomedical Engineering, State University of New York at Stony Brook, Stony Brook, NY, [2]Medicinal Chemistry and Center for Therapeutic Biomaterials, The University of Utah, Salt Lake City, UT, [3]Biomedical Engineering, Dermatology and Medicine, State University of New York at Stony Brook, Stony Brook, NY

Adult burn wounds, which lack hyaluronan (HA), often undergo excessive tissue remodeling and contraction. In contrast fetal wounds, which contain large amounts of HA, undergo remodeling that culminates in a scarless repair or regeneration. Therefore, adding a HA derivative to burn wounds would better mimic the fetal extracellular matrix (ECM) and may prevent excessive contraction. As a first step toward testing this hypothesis, we determined the effects of three HA moieties, i.e. high molecular weight (HMW)-HA, thiol-functionalized HA (HA-DTPH) and poly (ethylene glycol) diacrylate (PEGDA)-crosslinked HA (HA-DTPH-PEGDA), on fibroblast-mediated, collagen gel contraction. This assay has been widely used to mimic the in vivo wound contraction. Results showed that all the additives were cytocompatible. Interestingly, HMW HA facilitated collagen gel contraction while HA-DTPH weakly and HA-DTPH-PEGDA strongly inhibited contraction. The inhibitory effect was more

pronounced at higher concentrations of either HA-DTPH or PEGDA. However, varying the PEGDA MW showed no significant effect. The supernatant of contracted collagen-HMW HA gels contained greater amounts of HA, as compared to those containing HA-DTPH-PEGDA, suggesting that HMW HA facilitates fibroblast contraction of collagen gels by acting as a lubricant where it is effectively squeezed out of the gels. Crosslinking renders HA-DTPH-PEGDA the structural stability required to resist the fibroblast contractile forces. Such biomaterials that inhibit excessive contraction of collagen gels in vitro may also prevent severe wound contraction in vivo.

1114

### A Novel Surface Patterning Method for Protein-Cell Interaction Studies

M. P. Lynch,[1] S. R. Nettikadan,[1] A. Sethuraman,[1] J. C. Johnson,[2] E. R. Henderson[1,3]; [1]BioForce Nanosciences, Ames, IA, [2]Dept. of Microbiology, Des Moines University, Des Moines, IA, [3]Dept. of GDCB, Iowa State University, Ames, IA

A common technique for studying protein-cell interactions involves patterning an ECM protein or other signaling molecule onto a surface that is suitable for cell growth and subsequent analysis. Current methods of molecular surface patterning include bulk coating, photolithography, microcontact printing, and microarraying. Of these methods, only microarraying can facilitate multiplexed printing of different molecules on the same surface. Features created using pin-tool or ink-jet microarrayers are, however, many times larger than most cells. It was our objective to develop a flexible method of directly patterning multiple molecules onto a surface at the same (or smaller) spatial scale as a single cell. Briefly, we used an instrument (NanoArrayer™) equipped with microfabricated Surface Patterning Tools (SPTs) to draw multiple parallel lines of laminin and a negative control, bovine serum albumin (BSA), on a glass surface. Proteins were loaded into reservoirs on the SPT that connect to microfluidic channels that dispense liquid onto the surface via a process known as FEMTO (Fluidics Enhanced Molecular Transfer Operation). Laminin lines were 600 µm long, 5 µm wide, with a pitch of 40 µm. The BSA lines were 1000 µm long, 10 µm wide, with a pitch of 40 µm. Proteins were incubated on the surface overnight, then the surface was blocked and washed to prevent non-specific adhesion of cells. Adherent cells were cultured on the patterned surface and found to adhere specifically to the laminin lines. Subsequent labeling of the proteins with fluorescent antibodies confirmed their presence and identity. We have concluded that this technique is superior to existing surface patterning methods with respect to multiplexing, flexibility of pattern design, and spatial scale. Future studies using cell signaling molecules to direct differentiation or induce proliferation of individual stem cells hold great potential for human health.

1115

### Robust Differentiation of PC12 Cells and ES-D3 Murine Embryonic Stem Cells on Substrates Coated with Photo Reagents

T. J. Naqvi, G. W. Opperman, M. A. Lodhi; Diagnostics and Drug Discovery, SurModics, Inc., Eden Prairie, MN

In the past several years there has been a paradigm shift in developing synthetic biomaterials to grow cells or harvest cell products, primarily for applications in tissue culture, tissue engineering and regenerative medicine. For *in vitro* applications, physical and chemical properties as well as the structure of the substrate have been modified to closely mimic the natural microenvironment found *in vivo*. Cell attachment, proliferation, differentiation and cell-cell interaction are greatly affected by these physical and chemical properties of the substrate. We designed several experiments to test the effect of surface properties on the differentiation of PC12 and ES-D3 cells. PuraMatrix™ and Matrigel™ were used as substrates in addition to the substrates coated with photo-reagents containing 3-aminopropylmethacrylate (APMA), polyethylenimine (PEI), fibronectin, laminin, collagen and RGD peptides. Quantitatively better cell differentiation was achieved in PC12 cells grown on substrates coated with photo reagents compared to the other products: photo-laminin (80%) photo-APMA (60%), photo-collagen (30%), photo-PEI (20%) photo-RGD and PuraMatrix (10%), Matrigel (5%) and uncoated polystyrene control (1%). In addition, ES-D3 stem cells attach and divide well on surfaces coated with photo-APMA while remaining totipotent in the presence of FGF. With the proper change in growth conditions, cells differentiated into neurons, oligodendrocytes and type I and type II astrocytes. Moreover, photo-APMA proved to be an effective substrate in long-term maintenance and growth of the cells. PC12 and ES-D3 cells grown on APMA-coated substrate were robust and displayed their respective morphologies even after 60 and 30 days in cultures respectively, without being passaged onto fresh substrate. In conclusion, an APMA-coated surface provides a robust synthetic substrate for cell attachment, differentiation and long-term culture of the cells.

1116

### Probing the Role of Syndecan-Based Spreading on Cell Structure

J. Kubicek,[1] M. DiGiacomo,[2] A. Kamien,[2] P. LeDuc,[1] R. Bellin[2]; [1]Department of Mechanical and Biomedical Engineering, Carnegie Mellon University, Pittsburg, PA, [2]Department of Biology, Holy Cross College, Worcester, MA

Syndecans have been implicated in a wide variety of processes including both cell signaling and adhesion. Members of this superfamily of heparan sulfate proteoglycans are located at the cell surface and have known binding affinities for the extracellular matrix and the actin cytoskeleton. Because syndecans are linked to both the internal components of the cell and the external cellular environment, their transmembrane role in attachment and spreading is critical. Here, we present studies demonstrating our ability to control the spreading of NIH-3T3 fibroblasts by growing cells on modified polydimethylsiloxane (PDMS) membranes. These polymer membranes have been used in a variety of other cell studies to probe cell motility and mechanotransduction. In order to assay the effects of cell adhesion based on syndecans alone, we have developed a method to conjugate syndecan ectodomain-specific antibodies to the surface of PDMS membranes. After conjugating the syndecan antibodies to the polymer surface through plasma oxidation modification, cells were cultured on these membranes and allowed to attach and spread. Cells were then stained and visualized to probe the resulting cell structure including the actin cytoskeleton. The effects of controlling the concentration of the antibodies on cell spreading were also investigated. These results were compared to parallel studies on cell attachment and spreading using fibronectin conjugated PDMS, which is known to support cell adhesion via both syndecans and integrins. Overall, these studies have a variety of potential applications, which include studying cell structure and motility.

1117

### Forces Exerted by Epithelial Cells are Proportional to the Substrate Rigidity

B. Ladoux,[1] A. Saez,[1] A. Buguin,[2] P. Silberzan[2]; [1]University Paris 7-CNRS, MSC, Paris, France, [2]CNRS/IC, Institut Curie, Paris, France

We have developed a substrate composed of a closely spaced array of elastomeric microposts to measure traction forces exerted by cells on their

substrate (PNAS 2005). Previous studies have shown that cell movement and focal adhesions are regulated by physical interactions at the cell-substrate interface. We show that traction forces and focal adhesions are strongly correlated to the rigidity of the substrate by analyzing the force *versus* rigidity depending relation. We characterize quantitatively this effect on MDCK epithelial cells by using our micro-fabricated force sensor consisting of a high density array of soft pillars whose stiffness can be tailored by changing their height and radius. We find that the forces exerted by the cells are proportional to the spring constant of the pillars. Such a behavior supports the existence of a cellular integrated mechanism allowing cells to control the deformation of their substrate in response to a probing force. These dynamic observations are correlated with the reinforcement of focal adhesions that increases with the substrate rigidity.

1118

**Cell-Matrix Entanglement and Mechanical Anchorage of Fibroblasts in Three Dimensional Collagen Matrices**

H. Jiang, F. Grinnell; Cell Biology, UT Southwestern Medical Center, Dallas, TX

Fibroblast-3D collagen matrix culture provides a physiologically relevant model to study cell matrix interactions. In tissues, fibroblasts are phagocytic cells, and in culture they have been shown to ingest both fibronectin and collagen-coated latex particles. Compared to cells on collagen coated coverslips, phagocytosis of fibronectin-coated beads by fibroblasts in collagen matrices was found to be reduced. This decrease could not be explained by integrin reorganization, tight binding of FN beads to the collagen matrix, or differences in overall bead binding to the cells. Rather, entanglement of cellular dendritic extensions with collagen fibrils appeared to interfere with the ability of the extensions to interact with the beads. These extensions began to protrude as early as cells could be observed to interact with the collagen matrix and increased in length within the porous collagen network, eventually becoming difficult to distinguish by scanning electron microscopy from the collagen fibrils themselves. Entangled dendritic extensions were strongly anchored in the collagen matrix since treatment of attached cells with 0.25% trypsin/1mM EDTA caused breaks between the cell bodies and extensions rather than detachment of cells from the matrices. Similarly, although 10 mM EDTA inhibited cell adhesion to collagen matrices, once the cells were attached they no longer were sensitive to EDTA treatment even though cells on collagen-coated coverslips detached completely under similar conditions. These findings suggest that entanglement of dendritic extensions in the collagen matrix represents an integrin-independent form of adhesion. In conclusion, we suggest that cell-matrix entanglement provides a novel mechanism of cell anchorage not previously recognized and uniquely reliant on the three dimensional character of the matrix.

1119

**Mechanical Strain Enhances ECM Induced Cell Fate Determination and Promotes Osteogenic Differentiation of Human Mesenchymal Stem Cells Through the ERK MAPK Pathway**

D. F. Ward; Biology, Rensselaer Polytechnic Institute, Troy, NY

Human mesenchymal stem cells (hMSC) are a population of multipotent bone marrow cells, capable of differentiating through multiple lineages. In addition to bone, some of the other pathways lead to cartilage, fat, or smooth muscle. The role of mechanical strain in influencing the lineage specific cell fate determination and promoting osteogenic differentiation of hMSC is not known. Current research in our lab is focused on determining the effects of mechanical strain on extracellular matrix (ECM) induced osteogenic differentiation including the cell fate determination and passage through a specific lineage. We hypothesize that strain is transduced by specific cell surface transmembrane integrins to a MAPK signaling pathway cascade leading to the activation of transcription factor(s) which induce expression of osteogenic genes and result in increased extracellular matrix deposition and mineralization. Our data indicate that cells subjected to a 5% mechanical strain on COL-I coated silicone rubber membranes have increased expression levels of well-established osteogenic marker genes and reduced expression levels of marker genes from alternate lineages. Additionally, the extracellular matrix was shown to have increased levels of mineralization in response to mechanical strain. Our data suggests that the extracellular signal-related kinase (ERK) MAPK pathway is involved in this activation of bone lineage specific transcription factor(s) that control osteogenic gene expression during differentiation. These results suggest that mechanical strain enhances ECM induced cell fate determination and promotes osteogenic differentiation in hMSC through the ERK MAPK signal transduction pathway.

1120

**A Novel Platelet-endothelial Secreted Protein SCUBE1 is Present in Human Atheromas and Supports Platelet Adhesion via its Amino-terminal Epidermal Growth Factor-like Repeats**

R. Yang, C. Tu, Y. Su, B. Wu, M. Tsai; Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan Republic of China

We have previously identified a full-length cDNA encoding a secreted, membrane-anchored protein from human endothelial cells, termed SCUBE1 for signal peptide, CUB and epidermal growth factor-like (EGF)-like domain containing protein 1. Here, we further investigated its protein expression and function in the vasculature system. Immunohistochemical and flow cytometrical analyses demonstrated that SCUBE1 is an endogenous, surface-tethered endothelial protein that could be secreted as a soluble form and cleaved by a serum-associated protease. We also found that SCUBE1 is expressed in human platelets both at the mRNA and protein levels. Confocal microscopy and immunogold labeling revealed that while present at the surface, this protein is predominantly found in membranes of α-granules and elements of the open-canalicular system in the platelet. Interestingly, the platelet SCUBE1 is translocated to the surface upon thrombin stimulation and its immunoreactivity is localized within the extracellular matrix of human advanced atherosclerotic lesions. Studies of platelet adhesion to the surface-bound SCUBE1 fragments showed that only fragments containing the amino-terminal EGF-like repeats were able to support adhesion. We also found that plasma hSCUBE1 levels are significantly elevated in patients with acute coronary syndromes. Together, SCUBE1 protein released from platelets and/or the endothelium may contribute to the pathogenesis of atherosclerosis through its deposition and subsequent recruitment of activated platelets at the lesion sites.

1121

**Na,K-ATPase Mediated Cell-substratum Attachment Involves Fak and Src Signaling**

S. P. Barwe, S. A. Rajasekaran, A. K. Rajasekaran; Pathology and Laboratory Medicine, University of California, Los Angeles, Los Angeles, CA

Na,K-ATPase is a ubiquitous plasma membrane bound enzyme that is mainly composed of two non-covalently linked subunits, a 110 kDa α-subunit (Na,K-α) and a 55 kDa β-subunit (Na,K-β). Na,K-ATPase pump activity catalyzes an ATP-dependent transport of three sodium ions out

and two potassium ions into the cell per pump cycle, thereby generating a sodium gradient across the membrane. The Na,K-α is the catalytic subunit where as the Na,K-β plays a regulatory role and is required for the maturation of Na,K-α. Recently, we showed that Na,K-β is involved in the suppression of cell motility in carcinoma cells by a novel mechanism involving a cross talk between the two subunits of Na,K-ATPase and the members of the PI3-kinase signaling pathway. We found that Na,K-ATPase is localized to the lamellipodia, an actin rich microdomain generally found in cells with increased cell motility. In this study, we present evidence that decreased cell motility of Na,K-β expressing cells is due to increased attachment of these cells to the substratum. Furthermore, we found that activation of focal adhesion kinase by Src is involved in the increased attachment of Na,K-β expressing cells to the substratum. These results thus provide further understanding towards the mechanism of motility suppressor role of Na,K-β.

1122

**Light Chain 3 (LC3), a Microtubule Associated Protein Increases mRNAs Associated with Cell Adhesion**

L. Ying, M. Rabinovitch; Pediatrics, Stanford University, Stanford, CA

We reported increased migration of smooth muscle cells related to enhanced translation of Fibronectin (FN) mRNA, via an interaction between LC3 and an AU-rich element (ARE) in the 3' UTR of FN mRNA. To identify LC3 dependent functions in other cells, we investigated HT1080 fibrosarcoma cells that produce little FN and no detectable LC3 protein. Transfection of HT1080 cells with LC3 (WT-LC3) confirmed heightened FN mRNA translation by polysome analysis, and further transfection with a wild type (WT) and mutant rat FN mRNA, confirmed ARE dependence. We next compared WT-LC3 and vector-control cells with respect to invasion, and adhesion and determined whether these features were FN dependent. Using a matrigel assay, WT-LC3 cells were more invasive by 30% (n=8, P<0. 01) and adhesion to plastic was ~3-fold (n=3, P<0.002) higher than that of vector-control cells. Addition of FN to vector-control cells increased adhesion (p<0.001), to values approaching those in WT-LC3. However, addition of collagen IV or poly-D-lysine but not laminin similarly increased their adhesion. To determine whether enhanced FN mRNA translation was sufficient to explain the increased adhesion of WT-LC3 cells, we added CS-1 peptides and FN antibodies. This only resulted in a trend toward decreased adhesion, suggesting that factors additional to FN were to be considered. Microarray analysis of heavy polysomes in WT-LC3 vs. vector-control cells revealed significant increase in expression of genes associated with adhesion such as cdc42, a ras oncogene and small GTPase activating protein, and microarray analysis of total RNA revealed other genes confirmed by qRT-PCR to be higher in WT-LC3 cells but not induced by cell adhesion to FN, and these included thrombospondin-1, connective tissue growth factor and inhibin A. In conclusion, LC3 increases FN mRNA translation and modulates cell motility.

1123

**Second-hand Cigarette Smoke in the Eye: Stimulation of Inflammation and Delay in Corneal Re-epithelialization**

C. Ma, M. Martins-Green; Cell Biology and Neuroscience, University of California Riverside, Riverside, CA

Corneal re-epithelialization is a key process in preventing abnormal cornea wound healing. For example, delays in corneal re-epithelialization can result in pathogen invasion and their contribution to damage of the underlying stroma. Therefore, identification of factors that promote corneal re-epithelialization, and their mechanisms of action, will provide insights into improving and accelerating epithelial healing. Over the past two decades, it has been shown that environmental cigarette smoke delays skin wound healing, but very little is known about its effects on healing of cornea wounds. To address this question, we used mice that have been smoking for a prolonged period of time in an established system that mimics second-hand smoking by humans. Mechanical injury of the epithelium was done by removal of the corneal epithelium using a trephine and a scalpel. The mice continued to smoke during the healing process. We found that second-hand cigarette smoke delays the re-epithelialization process; the epithelium at the front edge of the wound does not adhere to the basal matrix and the cells show stronger F-actin staining. Immunolabeling shows that there is an accumulation of neutrophils in the wounded area in the smoking mice, and that many of the ECM molecules critical for epithelial cell migration are not present. Immunoblot analysis confirmed these findings. In addition, both, by immunoblotting and zymography, we find that MMP-7 levels are much higher in the wounds of the mice exposed to smoke. Furthermore, inflammatory cytokines, such as IL-1, TNF-alpha, and GRO-alpha increase in the corneal stroma and epithelium. These results suggest that second-hand cigarette smoke inhibits epithelial cell migration and enhances the inflammatory response during cornea wound healing, and that, in combination, these effects result in delayed corneal re-epithelialization.

1124

**Measuring pH in Very Close Proximity to the Outer Leaflet of the Plasma Membrane of Human Melanoma Cells**

C. Stock, A. Schwab; Institute of Physiology II, University of Münster, Münster, Germany

Migration of human melanoma (MV3) cells (i) is based on a coordinated formation and release of focal adhesion contacts mediated by integrin receptor molecules, (ii) requires the activity of the $Na^+/H^+$ exchanger NHE1 and (iii) depends on extracellular pH ($pH_e$). We favour the idea that, at focal adhesion sites, a local pH is created by protons extruded by the NHE1 together with protons provided by the extacellular solution. The local pH may then influence the strength of cell adhesion, i.e. of the integrin/extracellular matrix bond, and thereby modulate cell migration. In order to prove this hypothesis we developed an efficient method that enabled us to measure the pH in very close proximity to the plasma membrane ($pH_{em}$). MV3 cells were labeled with the phosphoethanolamine fluorescein DHPE. The labeled cells were continuously superfused with prewarmed HEPES-buffered Ringer solutions of various pH values. The fluorometrically determined $pH_{em}$ was slightly higher than the given $pH_e$ indicating that the glycocalix functions, at least conditionally, as a buffer. $pH_{em}$ decreased or increased as soon as $pH_e$ was lowered or elevated by changing the pH of the superfusion solution. Moreover, $pH_{em}$ of cells initially treated with the specific NHE1 inhibitor cariporide (HOE642) decreased by up to 0.1 units as soon as HOE642 was washed out. From these results we conclude that the fluorescein DHPE was incorporated only in the outer leaflet of the membrane. Thus, we were able to measure $pH_{em}$ locally at the focal adhesion sites located at the leading edges of lamellipodia.

## Extracellular Matrix & Cell Signaling II (1125-1149)

1125

### Role of Cell to Cell Signaling in Virulence of *Pseudomonas Aeruginosa* in Murine Model of Experimental Pyelonephritis

R. Mittal, S. Chhibber, S. Sharma, K. Harjai; Department of Microbiology, Panjab University, Chandigarh, India

Quorum sensing signals have been proposed to play most important role in the pathogenesis of respiratory tract and burn wound infections caused by *Pseudomonas aeruginosa*. This pathogen has been reported to monitor its cell density as well as expression of virulence determinants by quorum sensing signal mechanisms operative through autoinducers. However no reports are available in literature where role of these signals have been studied in relation to *P. aeruginosa* induced pyelonephritis. The present investigation was planned to study role of quorum sensing molecules in experimental pyelonephritis employing standard parent strain (PAO1) possessing functional quorum sensing systems (both *las* and *rhl*) and its isogenic mutant strains, single mutant deficient in production of *las*I and double mutant deficient in production of both *las*R and *rhl*R. It was observed that quorum deficient mutant strains were significantly less virulent as compared to quorum sensing producer parent strain during the course of infection assessed in terms of renal bacterial load and alteration in renal pathology. The present study brings out that cell to cell signaling plays most significant role in development of pyelonephritis caused by *P. aeruginosa.*

1126

### The Matrix Protein CCN1 Enhances FasL-Induced Apoptosis

V. Juric, C. C. Chen, L. F. Lau; Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago College of Medicine, Chicago, IL

The extracellular matrix protein CCN1 (CYR61) regulates diverse cellular functions through direct binding to integrins receptors. CCN1 expression is induced in tissue response to injury and in pathological contexts where angiogenesis and inflammation play central roles. The recent discovery that CCN1 can either induce apoptosis or protect cells from death in a cell type-specific manner prompted us to investigate whether CCN1 can modulate the cytotoxicity of known apoptotic inducers. One such factor is FasL, a member of the TNF family of inflammatory cytokines, which triggers cell death through the extrinsic pathway upon binding to its cell surface receptor Fas. Whereas no killing was observed in primary human fibroblasts treated with recombinant CCN1 alone, CCN1 significantly augmented FasL-induced cell death. CCN1 and FasL together induce apoptosis, rather than necrosis, as judged by flow cytometry following Annexin V/PI labeling. Pre-incubation of cells with function-blocking integrin antibodies and soluble heparin identified integrin $\alpha_6\beta_1$ and cell surface heparan sulfate proteoglycas (HSPGs) as receptors for CCN1 in this process. Consistently, mutant CCN1 proteins deficient in binding $\alpha_6\beta_1$ and/or HSPG failed to enhance FasL-induced apoptosis. CCN1 does not change the caspase requirement of FasL-mediated apoptosis, but is able to sensitize cells to FasL treatment. Pre-incubation of cells with CCN1 leads to a significant reduction in the time required by FasL to trigger cell death. In summary, the extracellular matrix protein CCN1 positively regulates FasL-induced apoptosis in fibroblasts. This is the first demonstration that FasL-induced programmed cell death can be enhanced by a matrix protein via an integrin and HSPG-dependent mechanism.

1127

### Cell Adhesion Regulates Serum Response Element Activity

C. Q. Cheng, D. M. Pirone, C. S. Chen; Department of Bioengineering, University of Pennsylvania, Philadelphia, PA

The Serum Response Element (SRE) is a key transcriptional regulator that links growth factor stimulation to many downstream functions including proliferation, differentiation, and migration. While the molecular pathways that link growth factor stimulation and SRE activity have been well described, in many contexts cell adhesion to extracellular matrix (ECM) acts as a critical cooperative component to growth factor signaling. Here we have examined the role of cell adhesion on SRE activity. To modulate cell adhesion, we seeded cells onto surfaces coated with different densities of the ECM protein fibronectin. Cells seeded on low density fibronectin attached but did not spread and flatten against substrates, and when exposed to serum these cells exhibited dramatically attenuated SRE activity compared to cells on the high density fibronectin substrates, as measured by luciferase reporter assays. Because altering fibronectin density modulates cell adhesion at several levels, affecting not only integrin ligation and clustering, but also cell spreading and flatterning against the substrate, we used micropatterned substrates to precisely and quantitatively modulate cell spreading without altering fibronectin density. On the micropatterned substrates, unspread cells were unable to transduce serum or growth factor signals into SRE activity. We have begun to characterize the molecular signaling pathways by which cell adhesion and spreading mediate SRE activity. Early results suggest that both ERK and RhoA signaling pathways are involved. These data highlight the importance of the adhesive microenvironment in transcriptional signaling and may yield insights into understanding the mechanisms by which adhesion and growth factor signaling pathways cooperate to regulate gene expression.

1128

### CD47-mediated Inhibition of Phagocytosis: Effects on Circulation Time of Polystyrene Microspheres in Mice

R. Tsai, D. Discher; University of Pennsylvania, Philadelphia, PA

CD47 reportedly inhibits the phagocytic clearance of red blood cells and platelets as shown in mouse. This inhibition is mediated by interaction between CD47 and its natural ligand, SIRPa present on macrophages. By removing CD47 from red blood cells it resulted in an increased clearance. However by using the knockout strategy employed may have caused other perturbations introduced to theses cells that may complicate the generalization of CD47 role. We propose animal experiments employing strategies to determine if the addition of CD47 to synthetic particles will inhibit their phagocytic clearance. Oriented immobilization of recombinant Ig-domain of mouse CD47 to polystyrene microspheres was performed using biotin-avidin chemistry. These modified microspheres were shown to bind to the extracellular domain of mouse SIRPa. Experiments are currently being conducted to clearly understand the effect of CD47 on the clearance of these microspheres in mice. The density will be varied in order to determine if there is a balance between adhesion and signaling. From these results we can demonstrate the role of CD47 in phagocytosis inhibition; paving the way for use in drug delivery for extending circulation times.

1129

**The Role of LIMD1 in Osteoclast Development and Function**

H. Epple,[1] Y. Feng,[1] R. Faccio,[2] G. D. Longmore[1]; [1]Medicine and Cell Biology, Washington University in St. Louis, St. Louis, MO, [2]Pathology and Immunology, Washington University in St. Louis, St. Louis, MO

Bone remodeling and homeostasis is a complex process requiring the balanced actions of bone forming osteoblasts and bone resorbing osteoclasts. Abrogation of either action leads to disorganization of the bone environment and osteoporotic or osteopetrotic phenotypes. The process of osteoclast differentiation is regulated by receptor activator of nuclear factor kappa B (RANK) signaling and requires formation of multinucleated cells with distinct apical basal polarity. The ruffled membrane at the basal surface of the osteoclast and an organized actin ring are required for efficient bone resorption. TRAF6 is an important signaling molecule downstream of RANK, promoting both the differentiation and resorptive functions of osteoclasts. The LIM protein, LIMD1, is present at chromosome 3p21, a locus that has been linked to bone density in humans. Here we show that LIMD1 interacts with TRAF6 to regulate RANKL stimulated osteoclast development. Like TRAF6, LIMD1 expression is upregulated during osteoclast differentiation. Genetic inactivation of LIMD1 in mice results in impaired osteoclastogenesis *in vivo* in the presence of excess RANKL, without affecting basal bone homeostasis. In developing osteoclasts, LIMD1 is an activator of AP-1 and thus, NFAT2 expression is inhibited in LIMD1 null cells. LIMD1 and other LIM proteins are important components of adhesion complexes and contribute to cell adhesion and migration. We observe that LIMD1 also localizes to sites of adhesion in mature osteoclasts including podosomes and the actin ring. Interestingly, LIMD1 null pre-osteoclasts have impaired adhesion to vitronectin and decreased migration in response to M-CSF in vitro. Src is a major regulator of osteoclast adhesion, migration, and bone resorption. LIMD1 null cells exhibit decreased Src activation in response to integrin engagement. In conclusion, these results suggest that LIMD1, in conjunction with TRAF6 regulates osteoclast development and may regulate osteoclast function by modulation of Src activity.

1130

**Signal to Polarize the *Drosophila* oocyte Involves Down-regulation of Dystroglycan**

J. Poulton, W. Deng; Biological Science, Florida State University, Tallahassee, FL

In the *Drosophila* oocyte, establishment of the anterior-posterior (AP) axis requires activation of the EGFR pathway in the posterior follicle cells (PFC) of the developing egg chamber. Therefore, the PFC are believed to provide a signal to the oocyte necessary for proper AP axis formation. The nature of this signal and the targets of EGFR activation in the PFC remain elusive. Here we demonstrate that one critical facet of this signaling event is down-regulation of Dystroglycan (DG), an extracellular matrix receptor, in the PFC. In support of this, we show that disruption of the EGFR pathway in the PFC results in failure to down-regulate DG in those cells, and more importantly, DG knockdown via RNAi can rescue an oocyte polarity defect caused by disruption of EGFR signaling in the PFC. In addition, we demonstrate that overexpression of DG in the PFC is capable of disrupting oocyte polarity. These findings indicate that the polarizing cue from the PFC to the oocyte utilizes an atypical signaling mechanism involving the down-regulation of DG.

1131

**A Role for Diastrophic Dysplasia Sulfate Transporter (DTDST) and Sulfation in Fibronectin Matrix Assembly**

L. L. Galante, K. Huang, J. E. Schwarzbauer; Molecular Biology, Princeton University, Princeton, NJ

Fibroblasts and many adherent cells produce and assemble the adhesive glycoprotein fibronectin (FN) into an extensively branched extracellular matrix. In cancer, the ability to deposit fibronectin into a matrix is often reduced or lost. Decreased FN assembly by the human fibrosarcoma cell line HT1080 is recovered by treatments that activate the integrins, inhibit MEK signalling, or stimulate transcription as with addition of the glucocorticoid dexamethasone. A microarray analysis was performed to determine genes whose expression changed in HT1080 cells upon induction of FN assembly by either MEK inhibition or dexamethasone stimulation. One of the genes, significantly upregulated by both treatments, was diastrophic dysplasia sulfate transporter (DTDST). DTDST is a transmembrane sulfate transporter, whose mutation in human patients results in reduced sulfate uptake by fibroblasts and histologically in an under-sulfation of cartilage proteoglycans. Proteoglycans are extensively sulfated on their glucosaminoglycan (GAG) side chains and these chains interact with several sites on FN. Chlorate and xyloside treatments, that inhibited sulfation or GAG chain synthesis, respectively, GAG degrading enzymes, and competition experiments using soluble heparin or chondroitin sulfate chains caused a reduction in FN matrix assembly. By contrast, excess soluble N- or O-desulfated heparin failed to inhibit matrix assembly as monitored by immunofluorescence microscopy and FN matrix insolubility in deoxycholate detergent. Thus, sulfated GAG chains are necessary for formation of a detergent-insoluble FN matrix. Blockade of DTDST expression with siRNAs also significantly reduced FN matrix, linking this sulfate transporter to the FN assembly process. Ongoing work is aimed at identifying a specific sulfated proteoglycan that is required for FN matrix formation.

1132

**Chondroitin Sulfate Proteoglycans are Involved in Modulating the Indian Hedgehog Signaling Pathway in the Mouse Growth Plate**

M. Cortes,[1] N. B. Schwartz[2]; [1]Biochemistry and Molecular Biology, U. of Chicago, Chicago, IL, [2]Department of Pediatrics, U. of Chicago, Chicago, IL

The functional role of heparan sulfate proteoglycans (HSPGs) in regulating signaling pathways such Hedgehog (Hh), Wingless-type (Wnt) and fibroblast growth factor (FGF) has been previously described. In contrast, the role of chondroitin sulfate proteoglycans (CSPGs) in these signaling pathways remains elusive. PAPS synthetase 2 (PAPSS2) is one of the two isoforms responsible for the synthesis of PAPS, the universal sulfate donor in all cells. The brachymorphic (bm) mouse is a PAPSS2 kinase domain mutant characterized by preferential undersulfation of CS chains and 50% reduction in limb length. *In situ* hybridization of the bm mouse growth plate with various markers, performed to determine whether signaling pathways regulating chondrocyte proliferation and differentiation are affected by undersulfation, revealed reduced mRNA levels of Indian hedgehog (Ihh), Patched. Immunostaining with hedgehog antibody (5E1) and Parathyroid hormone related peptide (PTHrP) antibody showed diminished extracellular diffusion of both signaling molecules in the bm mouse compared to wildtype. Consistent with reduction in Ihh and PTHrP protein, there was less cell proliferation in the bm mouse growth plate by PCNA antibody staining. In order to determine if CSPGs interact directly with Ihh, preliminary binding experiments with recombinant N-terminal Ihh and various forms of CS and HS chains revealed that both

HSPGs and CSPGs bind Ihh, with different affinities. In sum, undersulfated CSPGs may contribute to limb reduction in the bm mouse by affecting chondrocyte proliferation via the Ihh signaling pathway. .

1133

**CD44-hyaluronan Interactions Decrease FAK Activation**

J. I. Lopez, J. A. Schroeder; Molecular and Cell biology, The University of Arizona Cancer Center, Tucson, AZ

CD44 is a membrane bound adhesion molecule present on a variety of cell types, including mammary epithelial tumor cells. The role of CD44 in tumor progression and disease prognosis is a controversial one. While some believe CD44 to promote tumor progression, others believe that CD44 is not involved in tumor progression and may even be protective against invasion. We have recently demonstrated through the use of mouse models of breast cancer and CD44 null mice that CD44 expression confers protection from pulmonary metastatic disease while not affecting the growth of primary breast tumors. In the current study we examine mechanisms by which CD44 may impede cellular movement through extracellular matrices. The metastatic human breast cancer cell line MDA-MB-231 was allowed to invade into collagen gels cast in the presence or absence of 0.075µg/ml hyaluronan. CD44 was immunoprecipitated from invaded cells and western blots were probed for the presence and activation of coimmunoprecipitated signaling molecules. Preliminary results show that CD44 has decreased associations with activated FAK in the presence of hyaluronan, which may be indicative of decreased focal contacts in invading cells or increased apoptosis. In future experiments we will use Caspase-3 assays to measure cell death and imaging to examine cell morphology during invasion as well as the activation of other CD44 associated signaling molecules such as Src or ErbB2. Furthermore, constructs expressing the FAK inhibitor FRNK will be used to assess the functional significance of FAK activation in the context of CD44 signaling.

1134

**Ras Regulates Focal Adhesion Formation in 3D Matrices in a Manner Opposite of its Effects for on 2D Matrices**

M. A. Wozniak, P. J. Keely; Pharmacology, University of Wisconsin, Madison, WI

It is well established that activation of the small GTPases H-, N-, or K-Ras decreases focal adhesion formation. The Ras-induced inhibition of focal adhesion formation is commonly hypothesized to be advantageous in cancer, allowing cells to weaken their interaction with the extracellular matrix to promote migration and metastasis (Hughes et al., 1997). We also observe a decrease in focal adhesion formation in breast epithelial cells expressing activated K-Ras (12V). These cells show enhanced migration compared to control cells when plated on collagen-coated petri plates. In contrast, cells expressing activated R-Ras (38V), another small GTPase of the Ras superfamily, display enhanced focal adhesion formation and decreased cell migration. However, these experiments performed on 2D surfaces differ greatly from the *in vivo* 3D environment that cells normally encounter. When T47D cells are cultured in floating 3D collagen gels, they down-regulate focal adhesion formation and differentiate into duct-like tubules (Wozniak et al, 2003). We used this system to analyze how expression of oncogenes affects focal adhesion formation in a more relevant 3D environment. We find that expression of either activated K-Ras (12V) or R-Ras (38V) blocks tubulogenesis. Consistent with results in 2D culture, expression of activated R-Ras (38V) enhanced focal adhesions in cells cultured in 3D collagen gels. However, expression of activated K-Ras (12V) surprisingly also increased focal adhesion formation in cells cultured in 3D gels. These data demonstrate the significant differences between 2D and 3D culture. Furthermore, these results imply that in a more relevant 3D environment, K-Ras activation likely regulates signal transduction pathways differently, which has important implications for understanding the mechanisms by which oncogenes enhance invasion through complex matrices *in vivo*.

1135

**DDR1 Inhibits Collagen-induced Cell Spreading via Suppression of Cdc42 Activation**

Y. Yeh, M. Tang; Physiology, National Cheng Kung University, Tainan, Taiwan Republic of China

Discoidin domain receptor (DDR) is a receptor tyrosine kinase for collagen. We previously showed that over-expression of DDR1 in MDCK cells prevented cell spreading, whereas dominant negative DDR1 induced cell spreading trigger by collagen. However, the mechanism whereby DDR1 inhibits cell spreading has been unidentified. Cell spreading involves organization of actin cytoskeleton, which is mainly regulated by Rho family-GTPases. In order to examine whether Rho family-GTPases are involved in collagen gel-regulated cell spreading, we employed pull-down assay and transient transfection of constitutive active or dominant negative GTPases in MDCK cells and observed whether they could alter collagen-induced cell morphological changes. The results showed DDR1 decreased the activation of Rac1 and Cdc42, but had no effects on RhoA activity. Neither constitutive active nor dominant negative Rac1 could alter DDR1-inhibited cell spreading. However, constitutive active Cdc42 rescued the DDR1-inhibited cell spreading and dominant negative Cdc42 inhibited cell spreading in cells overexpressing dominant negative DDR1. These results indicate that DDR1-inhibited cell spreading is mediated by inactivation of Cdc42. With the use of 5E8, a potent $\alpha_2\beta_1$ integrin blocking antibody, we found that collagen-induced activation of Cdc42 is mediated by $\alpha_2\beta_1$ integrin. FRNK completely blocked collagen-induced decrease in Cdc42 activity, but DDR1 did not influence the phosphorylation levels of FAK. Taken together, DDR1 inhibits collagen-induced cell spreading by suppression of Cdc42 activation, which is mediated by $\alpha_2\beta_1$ integrin through FAK.

1136

**p120 is Required for Rac1-Induced Anchorage-Independent Cell Growth**

M. R. Dohn, A. B. Reynolds; Cancer Biology, Vanderbilt University, Nashville, TN

p120 expression is frequently reduced in human tumors, but the consequences *in vivo* are not yet well understood. To investigate the role of p120 loss in MDCK cells, we used siRNA techniques to knock down p120 and examined cell growth in soft agar, an assay generally used to predict tumorigenicity *in vivo*. Surprisingly, we found that p120 is required for Rac1-induced, but not H-Ras-induced, growth of MDCK cells in soft agar. p120 has previously been reported to inhibit RhoA in NIH3T3 cells. Similarly, we found that knock down of p120 in MDCK cells results in elevated RhoA activity. Inhibition of a downstream RhoA effector, ROCK, with the specific inhibitor Y-27632, rescues the ability of Rac1 to induce soft agar growth in the absence of p120. These data suggest that in MDCK cells, p120 plays an essential role in Rac1-mediated anchorage-independent growth by negatively regulating RhoA activity.

1137

**Role of RhoA/ROCK-Dependent Actin Contractility in the Induction of Tenascin-C by Cyclic Strain**

A. Sarasa-Renedo, V. Tunç-Civelek, M. Chiquet; ITI Research Institute, University of Bern, Bern, Switzerland

Objective: Here we explored the function of the actin cytoskeleton and of its RhoA/ROCK-controlled contractility in the induction of the extracellular matrix protein, tenascin-C, by cyclic tensile stress. Methods: Chick embryo fibroblasts were cultured on fibronectin coated silicone membranes and subjected to equibiaxial cyclic strain (10%, 0.3Hz). We have shown previously that this results in a 2-fold increase in tenascin-C mRNA within 6 hours; this response is attenuated by the ROCK inhibitor Y27632. Results: We now found that chemical activation of RhoA/ROCK by thrombin or LPA is sufficient to double the amount of tenascin-C mRNA in fibroblasts within 6 hours. When cells were pretreated with these drugs for 30 min, cyclic strain (6h) caused a super-induction (3.5-fold) of the tenascin-C mRNA level; the additional increase was again suppressed by Y27632. Microtubule disruption, which is known to trigger ROCK-dependent actin contraction, also induced tenascin-C mRNA. Conversely, disorganization of the actin cytoskeleton with latrunculin-A completely abolished induction of tenascin-C mRNA by either chemical RhoA/ROCK activators, mechanical stress, or both. Cyclic strain itself increased the amount of active RhoA in fibroblasts after 5 min as measured by a pulldown assay, and within 30 min triggered ROCK-dependent contraction of a collagen gel layer by the cells. Moreover, myosin II activity was shown to be required for tenascin-C induction by mechanical stress. Conclusions: From our results we conclude that RhoA/ROCK-mediated contractility of the actin cytoskeleton has a mechanosensory function in fibroblasts that relates directly to the expression level of the tenascin-C gene. Furthermore, we suggest that prior activation of actin contraction, by either chemical or mechanical signals, renders fibroblasts more sensitive to further external mechanical stress. This principle might be important in connective tissue regeneration and wound healing.

1138

**Substrate Stiffness Modulates Vascular Smooth Muscle Cell Response to PDGF: Involvement of Lipid Rafts**

X. Brown, R. Arjoon, J. Wong; Biomedical Engineering, Boston University, Boston, MA

More than 12 million Americans suffer from arterial occlusive disease. *In vivo* and *in vitro* studies have identified platelet-derived growth factor (PDGF) as the major stimulus for the abnormal vascular smooth muscle cell (VSMC) migration and proliferation during the development of the disease. However, therapies simply blocking the function of PDGF have limited success in clinical trials because the pathology of the disease is complex and highly dynamic. An engineered model system that recapitulates the biomechanical properties of the vessel was used to test the hypothesis that increased vessel stiffness during the development of the disease leads to an increase in the sensitivity of VSMCs to PDGF. The polyacrylamide based model system allows the stiffness to be systematically changed from 20 kPa to 80 kPa. while maintaining a constant ligand density of 5800 molecules of GRGDSP/ $\mu m^2$. With increased substrate stiffness, VSMCs have higher levels of FAK phosphorylation, increased cell-spread area, more defined stress fibers and increased rate of proliferation. VSMCs on stiffer substrate also have increased sensitivity to PDGF-BB stimulations. PDGF receptor is phosphorylated to higher levels in VSMCs on stiff substrate when exposed to the same concentrations of PDGF. This increased sensitivity is not due to increased protein level of PDGF receptor, but rather increased localization of PDGFR to the lipid rafts in the cell membrane.

1139

**Nanonewton Forces Applied to Individual Adhesions Causes Global, Dynamic Changes in Cellular Traction Forces**

M. T. Yang, N. J. Sniadecki, C. S. Chen; Department of Bioengineering, University of Pennsylvania, Philadelphia, PA

Mechanical forces exerted through the extracellular matrix are transmitted to cells, and transduced into biochemical responses through focal adhesions. A potentially important feedback loop may exist in which the mechanically-induced signaling could in turn regulate actomyosin-mediated traction forces, amplifying the mechanotransduction process. To measure the contractile forces of cells, our lab has developed a microfabricated array of elastomeric microposts which can report traction forces generated by cells spread across the posts. Here, we used a micromanipulator to direct a glass needle to deflect individual posts and therefore apply forces ranging from 15 to 32 nN to single NIH 3T3 fibroblasts, and subsequently measured their mechanical response. Early experiments indicate the presence of a baseline contractile tone in unperturbed cells, and that a step application of stretching force on peripheral adhesions causes a rapid, synchronized global loss of traction force. Within minutes the contractile tone in cells fell to nearly half of baseline levels. Under certain conditions, we observed a synchronized, global increase in contractility following this relaxation. We are currently identifying the molecular basis for these events. Understanding the role, if any, of these active mechanical changes in the biochemical response of cells to applied forces may provide insight into the mechanotransduction process.

1140

**Matrix Elasticity Directs Adult Mesenchymal Stem Cell Differentiation**

A. J. Engler,[1] M. F. Berry,[2] S. Sen,[1] H. Sweeney,[2] D. E. Discher[1]; [1]Biophysical Engineering Lab, University of Pennsylvania, Philadelphia, PA, [2]Physiology, University of Pennsylvania, Philadelphia, PA

Many different cell types respond to matrix stiffness as sensitively as more well studied soluble or immobilized ligands, yet mechanisms by which mechanical cues are transduced to cells have been far less explored. Here we demonstrate that expression of lineage markers in pluripotent mesenchymal stem cells (MSCs) requires not only growth factor stimulus but also proper matrix elasticity, $E$, in a myosin-dependent mechanism. Indeed, MSC morphology after one week, in the absence of growth factors, is highly mechano-sensitive; neuronal-like branched networks occur on soft matrices near $E_{\text{Nerve}}$ (<1 kPa), myoblast-like elongated morphology is observed at $E_{\text{Skeletal Muscle}}$ (8-17 kPa), and osteoblast-like polygonal morphology is observed on stiff matrices near $E_{\text{Osteoblast}}$ (>20 kPa). Oligonucleotide arrays of 120 neuronal, myogenic, and osteogenic genes show early marker expression for each MSC lineage on their specific matrices only, but western blots confirm that only partial expression of early markers relative to myoblast or osteoblast control cells; incomplete expression can be augmented by the addition of hydrocortisone or L-acetate-2-phosphate for myogenic or osteogenic commitment, respectively, thus inducing *full* lineage commitment; chemical or physical stimulus alone cannot. Mechano-senstive signaling for such differentiation pathways proves tension-based, involving non-muscle myosin II contractility: myosin II inhibition blocks lineage marker expression. However, similar myosin levels in myogenic differentiation factor 1- and core binding factor α1-expressing MSCs imply different Rho-induced tension-generating pathways, identified here as Rho GTPase signaling proteins mDia and ROCK,

respectively. Overall, this data implies that, in addition to chemical stimulus, tissue and/or matrix stiffness is critical for development.

1141

**Mechanical Stimulation of C2C12 Cells Increases m-calpain Expression and Activity, Focal Adhesion Plaque Degradation and Cell Fusion**

A. Grossi, A. H. Karlsson, M. A. Lawson; Department of Dairy and Food Science, Royal Veterinary and Agricultural University, Frederiksberg C, Denmark

Myogenesis is a complex sequence of events, including the irreversible transition from the proliferation-competent myoblast stage into fused, multinucleated myotubes. During embryonic development, myogenic differentiation is regulated by positive and negative signals from surrounding tissues. Stimulation due to stretch- or load-induced signaling is now beginning to be understood as a factor which affects gene sequences, protein synthesis and an increase in $Ca^{2+}$ infux in myocytes. Evidence of the involvement of $Ca^{2+}$ dependent activity in myoblast fusion, cell membrane and cytoskeleton component reorganization due to the activity of ubiquitous proteolytic enzymes known as calpains has been reported. Whether there is a link between stretch- or load induced signaling and calpain expression and activation is not known. Using a magnetic bead stimulation assay and C2C12 mouse myoblasts cell population, we have demonstrated that mechanical stimulation via laminin receptors leads to an increase in m-calpain expression, but no increase in the expression of other calpain isoforms. Our study revealed that after a short period of stimulation, m-calpain relocates into focal adhesion complexes and is followed by a breakdown of specific focal adhesion proteins previously identified as substrates for this enzyme. We show that stimulation also leads to an increase in calpain activity in these cells. These data support the pivotal role for m-calpain in the control of muscle precursor cell differentiation and thus strengthen the idea of its implication during the initial events of muscle development.

1142

**Human Type IV Collagen Induces STAT5 Activation in MCF7 Breast Cancer Cells**

L. A. Arriaga-Pizano, T. Robledo, M. Lopez-Perez, E. Perez Salazar; Biologia Celular, Cinvestav-IPN, Mexico DF, Mexico

Background/Aims. Extracellular matrix (ECM), including basement membrane (BM), is a complex network of interacting molecules such as collagens, fibronectin, and laminin. Signals from BM regulate morphology, growth, differentiation and apoptosis in mammary epithelial cells. The signal transducers and activators of transcription (STATs) can be activated by a number of cytokines, growth factors and ECM components. STAT proteins are normally involved in a variety of cellular processes. In particular, STAT5 plays a key role in mammary epithelial cell growth and differentiation. However, the participation of BM components in the activation of STAT5 in breast cancer cells, remain to be studied. Our aim was to study whether the BM component, type IV collagen (Col-IV), is able to induce STAT5 activation and the role of Src kinase family members activity. Methods/Results. By Western blotting with an anti-STAT5 antibody (Ab) and a phosphospecific Ab against the Tyr-694 of STAT5, electrophoretic mobility shift assay (EMSA) and the use of a Src kinase inhibitor (PP-2). We demonstrated that Col-IV induces phosphorylation of endogenous STAT5 at Tyr-694, nuclear translocation of STAT5 and an increase in STAT5-DNA complex formation in MCF7 cells. Treatment with the selective Src kinase inhibitor PP-2 inhibited all these effects induced by Col-IV. Conclusion Our findings delineate a signal transduction pathway from the cell surface to the nucleus in which Col-IV stimulation results in signaling through Src kinase to STAT5, which in turns translocates to the nucleus and binds to DNA. It remains to be determined the role of STAT5 activation in biological processes. This work has been accepted to publication in Matrix Biology. 2005

1143

**Collagen-induced Epithelial-to-Mesenchyme Transition in Pancreatic Cancer Cells**

Y. Shintani, K. R. Johnson, M. J. Wheelock; Oral Biology, University of Nebraska Medical Center, Omaha, NE

When epithelial cells change their relative position, they convert to motile fibroblastic cells, which is referred to as an epithelial-to-mesenchyme transition (EMT). We have shown that N-cadherin often becomes up-regulated in invasive tumor cell lines, which is critical to tumor invasion and metastasis. In addition, integrins play a central role in cell migration through their roles as adhesive receptors for ECM components. For migration in cancer cells, intercommunication between integrin-mediated cell motility and cadherin-based cell-cell adhesion is important. Our overall hypothesis is that integrin-mediated cell motility is coordinated with N-cadherin up-regulation through EMT-related signaling. BxPC-3 pancreatic cancer cells became flatter and more scattered, and showed increased N-cadherin, fibronectin and vimentin when cultured on collagen. Integrin ß1 inhibitory antibody (P4C10) prevented both the morphological changes and N-cadherin up-regulation. To elucidate signaling pathways active in collagen-induced EMT, we used signaling inhibitors such as SU6656 (SFK inhibitor), LY294002 (PI3K inhibitor), SP600125 (JNK inhibitor), SB203580 (p38MAPK inhibitor), and Y27632 (Rho kinase inhibitor). Only SP600125 inhibited collagen-induced cell scattering and N-cadherin up-regulation when BxPC-3 cells were cultured on collagen. These data suggest that integrin-JNK signaling is correlated with EMT and N-cadherin up-regulation induced by collagen. To establish a role for N-cadherin in collagen induced EMT, we generated N-cadherin over-expressing and knockdown BxPC-3 cells. Cell scattering was inhibited by N-cadherin knockdown. Furthermore, the transfectants that over-expressed N-cadherin were significantly more motile than mock-transfected cells in assays using transwell filters coated with collagen, while N-cadherin knockdown BxPC-3 cells showed a reduction in motility. Thus, we showed that N-cadherin up-regulation correlates with increased motility in collagen-induced EMT.

1144

**Focal Adhesion Kinase Signaling Pathways Regulate the Osteogenic Differentiation of Human Mesenchymal Stem Cells**

R. M. Salasznyk,[1] R. F. Klees,[1] A. Boskey,[2] G. E. Plopper[1]; [1]Biology, Rensselaer Polytechnic Institute, Troy, NY, [2]Mineralized Tissues Laboratory, Hospital for Special Surgery, New York, NY

The intracellular signaling events controlling human mesenchymal stem cell differentiation into osteoblasts are not entirely understood. We recently demonstrated that contact with extracellular matrix (ECM) proteins is sufficient to induce osteogenic differentiation of human mesenchymal stem cells (hMSC) through an extracellular signal-related kinase (ERK)-dependent pathway. We hypothesized that focal adhesion kinase (FAK) signaling pathways provide a link between activation of ERK 1/2 by ECM, and stimulate subsequent phosphorylation of the Runx2/Cbfa-1 transcription factor that controls osteogenic gene expression. To test this hypothesis, we plated hMSC on purified collagen I (COLL-

I) and vitronectin (VN) in the presence or absence of FAK-specific small inhibitory RNAs (siRNA), and assayed for phosphorylation of Runx2/Cbfa-1 as well as expression of established osteogenic differentiation markers (bone sialoprotein 2, osteocalcin, alkaline phosphatase, calcium deposition, and mineral:matrix ratio). We found that siRNA treatment reduced total endogenous FAK protein by ~40%, and reduced FAK phosphorylation on Y397 by 2.7-fold and 2-fold in cells plated for 30 minutes on COLL-I and VN, respectively. hMSC transfected with siRNA also exhibited a 3.3-fold and 3.8-fold decrease in ERK 1 phosphorylation on COLL-I and VN, respectively, after 1 hr. Serine phosphorylation of Runx2/Cbfa-1 was significantly reduced in treated cells. Finally, FAK inhibition blocked osteogenic differentiation of hMSC, as assessed by lowered expression of osteogenic genes (RT-PCR), decreased alkaline phosphatase activity, greatly reduced calcium deposition, and a lower mineral:matrix ratio after 28 days in culture. These results suggest that FAK signaling plays an important role in regulating ECM-induced osteogenic differentiation of hMSC.

1145

**Hyaluronan-CD44 Interaction with the Cytoskeleton and Signaling Molecules in Lipid Rafts Promotes $Ca^{2+}$ Mobilization, Oncogenesis and Tumor Progression**

L. Y. Bourguignon, A. Brightman, P. A. Singleton; Department of Medicine and VA Medical Center, University of California at San Francisco, San Francisco, CA

CD44 expression is closely associated with the progression of solid tumor cancers. The external portion of CD44 binds to an extracellular matrix (ECM) component [e.g., hyaluronan (HA)] while the intracellular domain interacts with cytoskeletal proteins and various signaling molecules. Our recent studies indicate that ankyrin, a cytoskeletal protein, and certain signaling molecules [e.g., Rho-Kinase (ROK) and PI3-kinase] are significantly enriched in CD44-containing lipid rafts [characterized as caveolin-rich plasma membrane microdomains] isolated from MDA-MB-231 cells (breast tumor cells), SK-OV-3.ipl cells (ovarian tumor cells) and SCC4 cells (HNSCC cells). This CD44 association with ankyrin and signaling molecules in lipid rafts is functionally coupled with HA-induced calciosome-cytoskeleton interaction, $Ca^{2+}$ mobilization, and tumor cell-specific behaviors (e.g., cell survival, growth and migration). Disruption of lipid rafts by the depletion of cholesterol from the raft membrane (using methyl-β-cyclodextrin treatment) not only abolishes the recruitment of ankyrin and various signaling molecules [e.g., Rho-Kinase (ROK) and PI3-kinase] into CD44-containing lipid rafts, but also impairs HA-mediated calciosome translocation, $Ca^{2+}$ signaling and tumor cell behaviors. These findings suggest that HA-CD44 interaction in lipid raft plays an important role in promoting ankyrin-based calciosome translocation/$Ca^{2+}$ signaling and activating intracellular signaling pathways (e.g., ROK-adducin signaling and PI3 kinase-AKT activation) leading to the onset of tumor cell-specific behaviors. These findings provide new insights into HA/CD44 interaction with the cytoskeleton and signaling molecules, which appears to regulate the malignancy of various human cancers including breast, ovarian and head/neck cancers.

1146

**Protein Tyrosine Phosphatases, Adhesion, and MEC Morphogenesis and Malignancy**

K. R. Johnson,[1] J. N. Lakins,[2] V. M. Weaver[2]; [1]Bioengineering, University of Pennsylvania, Philadelphia, PA, [2]Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Tumors, which are characterized by enhanced cell growth and adhesion-dependent invasion, exhibit a profound tissue desmoplasia that has been strongly implicated in malignant transformation. However, the molecular link between tissue desmoplasia and aberrant tumor growth and invasion remains poorly defined. Cell growth and invasion are regulated by crosstalk between integrins and growth factor receptors through protein tyrosine kinase signaling that is tempered by protein tyrosine phosphatase (PTP) activity. Although much is known about adhesion-dependent kinases, little information exists regarding links between PTPs and adhesion. Here, we explored the role of PTPs in adhesion-dependent invasion and growth using degenerate RT-PCR to amplify PTPs differentially regulated in mammary epithelial cells (MECs) in 2D versus reconstituted basement membrane (rBM)-differentiated mammary acini. We identified and validated the differential expression of several adhesion/morphogenesis-regulated PTPs and further characterized a subgroup of the Band 4.1 PTP family members as key regulators of normal and malignant MEC behavior. Using nonmalignant human MECs (HMT-3522 S-1 and MCF10A), we showed that during early mammary morphogenesis, when acini are rapidly forming, PTP-MEG1 and D1 mRNA expression is high, coincident with accelerated growth, actin reorganization, assembly of E-cadherin/β-catenin adherens junctions, and decreased matrix adhesion. Following morphogenesis, when adhesion remodeling and growth have ceased, MEG1 mRNA and protein expression is significantly repressed. Interestingly, malignant MECs (HMT-3522 T4-2) that fail to assemble adherens junctions and exhibit dynamic actin remodeling also show elevated and sustained levels of PTP MEG1 and D1. Consistently, ectopic expression of wild-type MEG1 dramatically enhanced matrix adhesion and accelerated growth of nonmalignant MECs within rBM. Because we found that MEG1 localizes to membrane-associated punctae, we are currently investigating a possible link between Band 4.1 PTPs, dynamic remodeling of adhesion/cytoskeletal complexes, and matrix-dependent MEC growth and morphogenesis. (Supp: DAMD17-03-1-0496, 5T32-HL007954-04, and HL6438801A1)

1147

**Inhibition of Laminin-5 Alpha 3 G4-5 Domain Selectively Disrupts Epidermal Tumorigenesis But Not Epithelial-mesenchymal Cohesion**

M. M. Tran,[1] P. Rousselle,[2] P. Nokelainen,[1] N. Nguyen,[1] E. F. Fincher,[1] M. P. Marinkovich[1]; [1]Epithelial Biology, Stanford University, Stanford, CA, [2]Chimie des Proteines, Institut de Biologie, Lyon, France

Laminin 5 is essential for epithelial-mesenchymal cohesion in many normal tissues, but also plays a critical role in epidermal tumorgenesis. Laminin 5 is secreted as a trimer (α3-β3-γ2) with subsequent processing of the α3 chain at residue 1337, removing the G4-5 domain. As a result, the G4-5 domain is largely absent in the basement membranes of fully developed normal tissues. However the G4-5 domain continues to be expressed in epidermal carcinomas. Our study sought to further characterize the role of the G4-5 domain in epidermal carcinomas. Retroviral transfer of full length (WTα3) or truncated (Tr1337) laminin α3 cDNA to laminin α3 null epidermolysis bullosa keratinocytes restored expression, trimeric assembly and deposition of laminin-5 in a polarized fashion. WTα3 cells transformed with Ras/IKB cDNA showed normal PI3-kinase activity, aggressive in vitro Matrigel invasion and significant in vivo tumor formation after SQ injection to nude mice. Ras/IKB transformed Tr1337 cells showed deficient PI3-kinase activity, and a lack of ability to invade Matrigel or form tumors in nude mice. G4-5 cDNA restored PI3-kinase activity and Matrigel invasion in transformed Tr1337 cells. G4-5 polyclonal antibody was shown to ablate tumor formation in mice injected

with normal Ras/IKB keratinocytes. Extensive necropsy analysis of multiple organs and electron microscopy demonstrated no epithelial adhesive defects and no basement membrane abnormalities associated with G4-5 pAb treatment. These results suggest that G4-5 domain of laminin α3 chain is essential for epidermal carcinogenesis and antibodies against the G4-5 domain represent an attractive potential cancer therapy.

1148

**The Activation of PKB/AKT Survival Pathway by Osteopontin in Prostate Cancer Cells**

B. Robertson, M. A. Chellaiah; Department of BMS, University of Maryland, Baltimore, MD

Osteopontin (OPN) is recognized as a key mediator of tumorigenesis and progression of cancer in experimental cell culture models as well as in clinical studies. Osteopontin interacts with $\alpha_v\beta_3$ integrins through its RGD domain. As an autocrine factor, OPN has been shown to increase cell motility and adhesion, affecting a wide range of down stream signaling molecules in osteoclasts. OPN/αvβ3-mediated signaling exhibited similar effects on actin cytoskelatal reorganization and migration in prostate cancer cells (PC3). Since, PC3 cells lack tumor suppressor, PTEN and OPN has been shown to be the cancer progression and survival factor in cancer cells, we analyzed the effects of OPN on the phosphorylation state of cell survival factor AKT. PC3 cells kept in serum-free medium were stimulated with OPN and mutated OPN (RGA). An increase in AKT phosphorylation was observed in PC3 cells treated with OPN and not in OPN (RGA). Similar increase was observed in PC3 cells over expressing OPN and not in PC3 cells over expressing mutated OPN (RGA). Inhibition of AKT phosphorylation by PI3-kinase inhibitor wortmannin suggests that PIP3-mediated membrane targeting of AKT/PKB as well as subsequent phosphorylation by the phosphoinositide dependent kinase-1 (PDK-1) may be required for the phosphorylation of AKT at Thr 308. *In-vitro* kinase assays reveal that OPN activation of the Akt phosphorylation at Thr-473 is ILK mediated while ILK inhibitor (KP392) analysis substantiate this observation. Our observations suggest that partial activation and phosphorylation by phosphoinositide dependent kinase -1(PDK1) on Thr 308 and subsequent phosphorylation by ILK at 473 may be required for the activation and phosphorylation of PKB/AKT.

1149

**Human Uterine Leiomyoma-Derived Fibroblasts Promote Proliferation of Uterine Leiomyoma Cells in a Coculture System**

L. Yu,[1] A. B. Moore,[1] C. D. Swartz,[2] X. Zheng,[1] L. Wang,[1] G. E. Kissling,[3] D. K. Walmer,[4] S. J. Robboy,[5] D. Dixon[1]; [1]Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences, RTP, NC, [2]Environmental Carcinogenesis Division, US Environmental Protection Agency, RTP, NC, [3]Biostatistics Branch, National Institute of Environmental Health Sciences, RTP, NC, [4]Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC, [5]Department of Pathology, Duke University Medical Center, Durham, NC

Uterine Leiomyomas (fibroids: myomas) are the most common benign tumors diagnosed in women. Fibroids consist of benign smooth muscle cells and an extracellular matrix (ECM) component. We hypothesize that interactions between the smooth muscle tumor cells and fibroblasts of the ECM are important in the growth of fibroids, and in the production of ECM proteins. In this study, we used a coculture system to determine the effects of human uterine leiomyoma-derived fibroblasts (FB) on the growth of uterine leiomyoma cells (UtLM), the induction of growth factors (GFs), the activation of GF receptors, and the production of ECM. Cell counts and proliferating cell nuclear antigen staining revealed increased proliferation of cocultured UtLM compared to UtLM cultured alone. As determined by ELISA or Western blot analysis, there was a significant increase (p=0.03 or less) in the secretion of TGF-β1, TGF-β3, VEGF, EGF, FGF-2, and PDGFα/β in the coculture media compared to media of UtLM and FB cultured alone. The TGF-βI receptor from cocultured UtLM cell lysates had increased phosphoserine and downstream phospho-Smad2 protein levels compared to non-cocultured UtLM. There was increased expression of phosphorylated protein tyrosine kinase (pPTK) receptors for VEGF, FGF-2, PDGFα/β, and downstream effector protein phophorylated 44/42MAPK confirming that these GFs were signaling through tyrosine kinases. There was a significant increase (p=0.03) in collagen-I, IGF-BP3 and IGF-BP4 in the media of UtLM cocultured with FB; this was not seen in FB cocultured with normal myometrial cells. In summary, tumor-derived FB stimulate the growth of UtLM, most likely by increased induction of GFs and activation of their receptor signaling pathways, and by enhanced production of ECM. These data support the role of GFs and ECM in the pathogenesis of fibroids.

## Cell-Cell Interactions II (1150-1163)

1150

**Cell Confluence-Induced Activation of Stat3 Triggers Dome Formation via Augmentation of NHE3 Expression in MDCK Cells**

H. Su, M. Tang; Physiology, Institute of Basic Medical Science, National Cheng-Kong University, Tainan, Taiwan Republic of China

The signal transducers and activators of transcription 3 (Stat3) is generally activated by cell confluence and cell-cell adhesion. However, the biological implication and associated regulatory mechanism remain unclear. Our study found that the activation of Stat3 by cell confluence preceded the dome formation in Madin-Darby canine kidney (MDCK) cells. Here we sought to elucidate how Stat3 activation triggered dome formation, particularly which transepthelial transport system was involved. Immunofluorescence and confocal microscopy studies showed that Stat3tyr705 was phosphorylated at the pre-dome forming area and the nuclei of dome forming cells, suggesting a tight association of nuclear signal of Stat3 tyr705 phosphorylation with dome formation. To further elucidate the relationship, we established MDCK cell lines over-expressing inducible active form of Stat3 (Stat3-C) or dominant negative Stat3 (Stat3-D). Dome formation was promoted by expression of Stat3-C and inhibited by Stat3-D. Moreover, levels of NHE3 but not NHE1 were increased in confluent cells. Interestingly, NHE3 expression levels could be up-regulated by Stat3-C and inhibited by Stat3-D, whereas NHE1, $Na^+$, $K^+$-ATPase or ENaC were not affected by Stat3 expression. Application of NHE3 inhibitors, EIPA and HOE694, could suppress confluence-induced dome formation. These results strongly indicate that activation of Stat3 by cell confluence triggers dome formation through regulation of NHE3 expression.

1151

**Heterocellular Communication Enhances Mammary Epithelial Cell Differentiation**

M. E. El-Sabban,[1] R. Mroueh,[2] M. Mokaled,[2] R. Talhouk[2]; [1]Human Morphology, American University of Beirut, Beirut, Lebanon, [2]Biology, American University of Beirut, Beirut, Lebanon

The link between gap junction intercellular communication (GJIC) and mouse mammary epithelial cell differentiation in vitro is investigated. SCp2

mammary epithelial cell clone differentiate in a basement membrane and hormone dependent manner. In the absence of exogenous basement membrane components, heterocellular interaction between the SCp2 epithelial and the SCg6 myoepithelial cell clones is critical for differentiation. We have recently demonstrated that the expression of connexin 30 correlates with the course of lactation in the gland. The expression profile of Cx30 is concurrent with SCp2 epithelial cell differentiation in vitro. Furthermore, connexins 30, 32, 43 associations with junctional proteins, catenins and Zonula Occludens, are enhanced in differentiation-permissive conditions, especially in heterocellular co-cultures of SCp2 and SCg6. These findings corroborate the importance of heterocellular gap junctions in mammary differentiation.

1152

**Differential Sub-cellular Localization of NGEP, a Prostate Specific Protein in Two Prostate Cancer Cell Lines**

S. Das, S. Nagata, T. Bera, I. Pastan; Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD

NGEP, New Gene Expressed in Prostate, identified through EST clustering is expressed only in normal prostate and prostate cancer. It is encoded in chromosome 2 on the human genome and there are two alternative splicing products (NGEP-L and NGEP-S). The long transcript of NGEP (NGEP-L) encodes a protein of 933 amino acids. Our previous study using the transient expression of the myc-epitope tagged NGEP-L in 293T cells shows that NGEP-L is a transmembrane protein expressed on plasma-membrane. Because of its exclusive expression in prostate and prostate cancer and also its cell surface localization, NGEP is a promising target for immunotherapy. In this study, we evaluated the effect of forced expression of NGEP on two prostate cancer cell lines, PC3 and LnCaP. Immunofluorescence using NGEP antibody revealed that NGEP is differentially localized in these cell lines. In case of LnCaP, NGEP is localized in the plasma membrane of the cell and preferentially in the cell-cell junction regions of the plasma-membrane. In contrast in PC-3 cells, intracellular accumulation of NGEP could be observed. Dual labeling of the PC3 cell with cis-golgi network antibody shows that NGEP is co-localized with the cis-golgi network. In PC3 cells, NGEP was also located in the cell surface derived endocytic vesicles, suggesting intracellular accumulation of NGEP is not due to the impaired trafficking to the cell surface. Macroscopically, expression of NGEP in LnCaP cells tended to form clumps. However in case of PC3 cells, the morphology was similar to that on the control cells. These results confirm that NGEP is a cell surface protein and suggest it might a have a role in cell contact- dependent interactions in the epithelial cells of the prostate.

1153

**Generation of a Functional Model for Normal Intestinal Epithelial Cell Differentiation**

C. R. Lussier, F. Boudreau; Anatomy and Cell Biology, Université de Sherbrooke, Sherbrooke, PQ, Canada

INTRODUCTION: As an ongoing renewal system in which proliferation, differentiation, migration and apoptosis are tightly regulated, intestinal epithelium is an excellent model to study these cellular processes at the molecular level. However, normal intestinal epithelial cell model able to recapitulate these processes in culture is not yet available. The aim of this work was to produce a normal model of intestinal epithelial cell differentiation. METHODS AND RESULTS: An IEC-6 cell line that stably expressed Cdx2 was seeded on a monolayer of human fetal intestine fibroblasts. Co-cultures were maintained for 12 days. At day 4 after seeding, up-elevated structures appeared and multiplied through day 12. Electron microscopy revealed that these structures consisted in a monolayer of polarized epithelial cells presenting characteristics of differentiated enterocytes such as brush border and all types of cell-cell junctions. Western analysis showed an up-regulation of Cdx2, GATA-4 and HNF 1$\alpha$, transcription factors known as regulators of specific intestinal genes associated with differentiation such as sucrase-isomaltase (SI). At day 12, SI mRNA expression was detectable by RT-PCR analysis. Moreover, an important matrix deposition between the epithelial and the fibroblastic cell layers was observed by electron microscopy. This matrix accumulation corresponded to an over expression of collagen IV $\alpha$1 chain by fibroblasts as demonstrated by RT-PCR analysis. CONCLUSION: Our results suggest that IEC-6 cells expressing exogenous Cdx2 are able to normally differentiate in a co-culture system with human intestinal fibroblasts. This model will be utilized to investigate molecular mechanisms involved in intestinal epithelial cell differentiation. Furthermore, the heterologous nature of the two cell compartments will make this co-culture system useful for epithelial-mesenchymal interactions studies.

1154

**Search of the Instrumental Feedback Mechanism in Biochemical Networks: A simple Linear Approach**

J. Ferreira,[1] S. Mondié,[2] R. Lozano,[1] A. Friboulet[3]; [1]Heuristique et Diagnostic des Systèmes Complexes (HEUDIASYC) UMR-CNRS 6599, Université de Technologie de Compiègne, Compiègne, France, [2]Department of Automatic Control, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), México, Mexico, [3]Laboratoire de Génie Enzymatique et Cellulaire, UMR-CNRS 6022, Université de Technologie de Compiègne, Compiègne, France

**Motivation:** The comprehension of several phenomena and its physiological significance in complex networks of living cells is a challenge for scientists from different fields of science. Experimentalist and theoretician are summoned to join their interdisciplinary efforts with view to contribute to the understanding of the complexity in biology. **Objective:** We analyse interactions in a dynamic model of a biochemical network driving the cell division cycle in Xenopus eggs in order to determine the structural components and interconnections that cause the complex behaviour. **Method:** We propose and implement a numerical solution for the system of nonlinear ordinary differential equations that represents the model. A linearization technique is used to approximate the system's behavior in the vicinity of the equilibrium point. A small perturbation is introduced in a given element of the Jacobian matrix and then a interactive algorithm is used to compute the smallest absolute value for which the stability/instability is induced. Bifurcation diagram is used to sketch special points considered useful in the analysis of system's eigenvalues. **Results:** Qualitative assessment of the results indicates the three most important proteins among the nine studied. Our results are confronted with a recent work of a different author who utilizes other technique and same mathematical model. The comparison shows that the two approaches point out the same three proteins as responsible for the phenomena. **Conclusions:** *in silico* experiments demonstrates that the proposed technique is a very useful alternative in identification of the key proteins and interactions that govern the oscillations in the Xenopus frog egg at high values of the rate of cyclin synthesis. This verification highlights that computer simulations associated with system theory constitute a powerful strategy in the comprehension of a biological systems as a whole.

1155

**TGF-Beta Effects on Desmosomal Components in MCF10A Cells**

S. A. Keim, K. R. Johnson, M. J. Wheelock; Oral Biology, University of Nebraska Medical Center, Omaha, NE

TGF-β Effects on Desmosomal Components in MCF10A cells. Epithelial to mesenchymal transition (EMT) is a normal part of embryonic development, and is a potential mechanism in carcinoma progression. During EMT, epithelial cells decrease cell-cell interactions and acquire an invasive phenotype. Epithelial cells interact with one another via several junctional complexes including the adherens junction and the desmosome. Changes in the adherens junction have been characterized during EMT, but desmosomes have received only limited attention. Our hypothesis is that, in addition to the known alterations in cadherin-mediated adherens junctional adhesion, MCF10A cells, which undergo TGF-β induced EMT, also alter their desmosomes. Punctate staining of desmoplakin at cell borders is used as a marker of desmosomes. Immunofluorescence of desmoplakin in MCF10A cells shows punctate cell-cell border staining in the control cells, whereas after treatment with TGF-β it becomes cytosolic. This change in desomoplakin implies desmosomes are being disassembled during EMT. Immunoblot analysis reveals that desmocollins, desmogleins, plakophilins, and plakoglobin all decrease after four days of TGF-β treatment. However, the behavior of these proteins is not uniform. Both plakoglobin and desmocollin 2 increase within the first two days, and then decrease beginning at three days of treatment with TGF-β. In contrast, desmoglein 2 and 3 and plakophilin 2 and 3 steadily decrease with TGF-β treatment. mRNA levels of these proteins do not decrease with TGF-β treatment suggesting that the mechanism for the decrease in protein levels is due to a posttranscriptional mechanism. These data characterize the disassembly of desmosomes after treatment with TGF-β and point to a posttranscriptional modification of the desmosomal proteins.

1156

**Nitric Oxide Induces Formation of Nanometer Membrane Tubulovesicular Extensions in Neutrophils, Establishing Cell-Cell Contacts Between Neutrophils and Binding Erythrocytes and Bacteria over a Distance**

S. I. Galkina,[1] Y. M. Romanova,[2] G. F. Sudina[1]; [1]Moscow State University, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow, Russian Federation, [2]The Gamaleya Research Institute of epidemiology and microbiology RAMS, Moscow, Russian Federation

Nitric oxide is known to reduce integrin-dependent (but not selectin-dependent) leukocyte adhesion to endothelial cells and to relief endothelium damage induced by adherent leukocytes upon ischemia/reperfusion or sepsis. Previously we have shown that neutrophils (polymorphonuclear leukocytes) possess a capacity to develop highly dynamic thin and long (several cell diameters in length) tubulovesicular extensions, connecting cells to substrata in β1-, β2-integrin-independent, but L-selectin-dependent manner. Recently we found that nitric oxide donor diethylamine NONOate caused appearance and growth of similar in size and behaviour tubulovesicular extensions in neutrophils upon adhesion to fibronectin-coated substrata. In the presence of nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester neutrophils spread on fibronectin and had no microextensions. Scanning electron microscopy revealed that diethylamine NONOate-induced extensions connected neutrophils over a distance and have the uniform diameter along the entire length. Flexible and strait extensions connected cells. Diameter of strait (tubular) extensions varied from 125 to 160 nm, diameter of flexible (consisted of tubular and vesicular fragments of the same diameter) varied from 180 to 290 nm. Strait extensions could be derived from flexible ones under tension. Tubulovesicular extensions of neutrophils also effectively bound and kept serum opsonized bacteria (*Salmonella typhimurium*), thus making them available for bactericidal substances secreted by neutrophils extracellularly. Leukocytes are known to scavenge old erythrocytes from the blood stream by phagocytosis. Neutrophil extensions induced by diethylamine NONOate bound erythrocytes over a distance and increased the number of bound erythrocytes for neutrophil. Membrane tubulovesicular extensions are supposed to represent protrusions of an intracellular exocytotic trafficking. Nitric oxide seems to impair fusion of exocytotic trafficking with the plasma membrane, thus inducing development of tubulovesicular extensions altering neutrophil contact interactions with other cells.

1157

**Repatterning the T Cell Immunological Synapse with Nanofabricated Supported Membranes**

A. L. DeMond,[1,2] K. D. Mossman,[1,3] B. Rossenova,[1,2] J. P. Hickey,[2] M. L. Dustin,[4] J. T. Groves[1,2,3]; [1]Biophysics Program, UC Berkeley, Berkeley, CA, [2]Chemistry, UC Berkeley, Berkeley, CA, [3]Physical Biosciences Division, Lawrence Berkeley National Labs, Berkeley, CA, [4]Pathology Dept., New York University, New York, NY

Spatial regulation of signaling is emerging as a dominant paradigm in intercellular communication. The junction between a T cell and an antigen presenting cell, called the immunological synapse (IS), has been established as a site rich in spatially regulated molecular interactions. Using total internal reflection fluorescence (TIRF) microscopy, we have visualized the movement of small clusters of T cell receptor (TCR) on the surface of T cell blasts during IS formation as they interact with planar supported bilayers displaying mobile peptide-major histocompatibility complex (pMHC) and intracellular adhesion molecule (ICAM-1). We have developed a technique using nanofabricated lateral constraints on the substrate to frustrate molecular motion in the interacting T cell (K.D. Mossman et al., *submitted*). On unpatterned substrates, TCR clusters were seen to migrate centripetally. The introduction of lateral constraints, such as grids, interrupted centripetal TCR motion, causing the clusters to change direction and track along the grid lines at a reduced velocity. We conclude that TCR transport is active and is restricted by the lateral constraint of its ligand, pMHC.

1158

**Modeling TCR Mobility and T Cell Repolarization**

O. Dushek, D. Coombs; Mathematics, The University of British Columbia, Vancouver, BC, Canada

T cells adhere to antigen presenting cells bearing appropriate antigenic stimuli, forming immunological synapses - well defined regions enriched in certain surface receptors and signaling molecules. In experiments where a T cell forms a synapse with a particular antigen presenting cell, and then encounters a second cell presenting a higher antigen load, the synapse reorients towards the second cell and eventually may detach from the first, over the course of several minutes. The receptors detecting antigen are T cell receptors, which are known to be mobile on the cell surface from FRAP experiments. We use computational modeling to show that thermodynamics and pure diffusion of cell surface receptors fails to explain the data. Directions for future exploration of this problem are indicated.

1159

**Analysis of Mice Lacking p120ctn or Small GTPases Expression in Hepatocytes**

J. van Hengel,[1] C. Van den Broeke,[1] P. D'hooge,[1] X. Wu,[2] A. Reynolds,[3] C. Brakebusch,[2] F. van Roy[1]; [1]Molecular Biomedical Research, VIB - Universtiy of Gent, Zwijnaarde - Gent, Belgium, [2]Junior group "Cytoskeletal Organization", Max Planck Institute for Biochemistry, Martinsried, Germany, [3]Department of Cancer Biology, Vanderbilt University, Nashville, TN

Analysis of Mice Lacking p120ctn or Small GTPases Expression in Hepatocytes Acquisition of invasive and metastatic capabilities is frequently associated with loss of cell-cell adhesion. The major constituents of the cell-adhesion complex in adherens junctions are cadherins, transmembrane proteins involved in homotypic cell-cell adhesion. The p120 catenin (p120ctn) protein is a member of the Armadillo protein family and a key component of adherens junctions, where it interacts with the juxtamembrane part of the cytoplasmic tail of several cadherins. Cytoplasmic p120ctn promotes cell motility, and probably also other activities, through inhibition of RhoA and activation of Rac1 and Cdc42. We generated mice lacking p120ctn expression in hepatocytes. Mutant mice were born at Mendelian ratios. Their phenotypic analysis is ongoing. The small GTPase Cdc42 regulates many different aspects of cell polarity, such as axon formation in neurons, orientation of the Golgi apparatus in the direction of migration, and directed secretion to the basal side of epithelial cells. To understand the role of Cdc42 in the establishment and maintenance of cell polarity in vivo, we generated mice lacking Cdc42 expression in hepatocytes. The regular liver plates were completely absent at age 2 months. The normally tight contacts between neighboring hepatocytes were often widened and the E-cadherin expression pattern was changed. Analysis of serum samples indicated a cholestatic phenotype characterized by at least fourfold elevated levels of AP, tenfold increased concentrations of total and conjugated bilirubin, and increased activities of GPT/GOT. Further analysis of the in vivo roles of p120ctn and Rho GTPases in the liver, and the relevance of their loss to the causation of hepatocellular carcinoma (HCC), one of the most common solid tumors worldwide, is underway.

1160

**Primary Neural Culture to Identify the Factors Controlling the Differentiation of Ependymal Cells**

N. Spassky; Inserm UMR-711, Hopital Pitié Salpetriere, Paris, France

In adult mammals, the cerebral ventricles are normally lined by a layer of ependymal cells. Most of these cells are cuboidal and multiciliated. These cells are at the interface between the brain parenchyma and the ventricular cavities and play an essential role in the propulsion of CSF through the ventricular system. During embryogenesis, we have previously demonstrated that they are derived from radial glial cells. The aim of our study is to identify the cellular and molecular mechanisms governing the differentiation of ependymal cells. We have set up an in vitro assay in which radial glial cells from the lateral walls of the lateral ventricles of newborn mice are purified to 98%, and allowed to differentiate in different culture conditions. After 10 days in culture in fœtal bovine serum (FBS)-free medium, 70% of radial glial cells differentiate into multiciliated ependymal cells, compared to 20% in FBS containing medium. To further investigate the putative roles of soluble secreted factors on the differentiation of ependymal cells, purified radial glial cells were cultured in FBS-free medium which was either renewed every 2 days to dilute all secreted factors or left unchanged. In both cases, 70% of radial glial cells differentiate into multiciliated ependymal cells after 10 days in culture. We obtained similar results in the presence of a gap junction blocking agent (carbenoxolone), perhaps suggesting that neither contact or soluble secreted factors induce the differentiation of ependymal cells in our culture model. We are now in the process of testing the influence of other contact factors such as notch/delta and BMP signalling on the differentiation of ependymal cells.

1161

**Studies of Cytoskeleton during the Interaction of *Sporothrix schenckii* - Epithelial cells**

M. Sabanero López,[1] G. Sandoval,[1] A. Carabez,[2] J. C. Villagómez,[1] E. López-Romero,[1] H. Torres- Guerrero[2]; [1]Instituto de Investigación en Biología Experimental, Universidad de Guanajuato, Guanajuato, Mexico, [2]Instituto de Neurobiología, Universidad Nacional Autónoma de México, México City, Mexico

Adherence of pathogenic microorganisms to host tissues is regarded as a pre requisite for dissemination. Microbial adherence has been studied extensively in pathogenic bacteria and fungi such as *C. albicans*, *A. fumigatus*, and *B. dermatitidis*, but little is known about the adherence mechanism in *S. schenckii*. This study was undertaken to examine the cytoskeleton in the host cell during the interactions and invasion of *S. schenckii* strains MP - 109. We found that yeast ($10^5$ cells /ml) and mycelium ( 100µl/ml) adhered and invaded (6 to 24 h) culture human cervix epithelial cells. The invasion abilities of mycelium were much hight that the yeast. Immunofluorescence microscopy studies showed that the adherence of mycelium and yeast induced cytoskeletal alterations. Under the adherent sites calcofluor studies showed an intimate association between cell wall of fungus and epithelial cells. These results indicate that surface of fungus plays important roles in the invasion of epithelial cells, and the alterations of cytoskeleton in host cell for invasion. The work was support by the Universidad de Guanajuato. (Grant No. 18 to MSL)

1162

**Cytogenetic Mechanism of Cytoplasmic Incompatibility in the Uzifly, *Exorista sorbillans* (Diptera: Tachinidae)**

H. P. Puttaraju, B. M. Prakash; Sericulture, Bangalore University, Bangalore, India

Vertically transmitted bacteria of the genus *Wolbachia* are responsible for the early death of embryos in crosses between *Wolbachia* infected males and uninfected females in a number of insect species studied to date. This mechanism is known as unidirectional cytoplasmic incompatibility. This mechanism when occur between males and females harboring different strains of *Wolbachia* termed as bi-directional cytoplasmic incompatibility. The *Wolbachia* is found in the uzifly, *Exorista sorbillans*, a serious endoparasite of silkworm, *Bombyx mori* L, express both the types of cytoplasmic incompatibility (CI). In the present paper, we investigated cytological mechanism of CI. The results revealed that, in crosses between infected males and infected females when the sperm entered the egg, the sperm chromatin condenses to form the paternal pronucleus. Subsequently, the chromosomes condense for mitosis and spindle attachment occurs and then paternal and maternal pronucleus fuses to form the diploid zygote. In eggs from crosses between infected males and uninfected females only the maternal pronucleus forms individual chromosomes. Whereas the paternal pronucleus does not condense in individual chromosome, but scattered near maternal pronucleus and in some eggs reappear as a diffuse tangled chromatin mass and tend to get fragmented during the first mitotic division thus, the failure of syngamy. However, abnormal behavior of paternal chromatin does not interfere with mitotic division of maternal chromosomes. From these result it is evident that the *Wolbachia* induced CI leads to chromosome destruction and subsequent death of embryo. The mechanism is now implicated widely in the management of

medical and agricultural important pests.

1163

### A 3-D Model to Study Interplay of Cell Adhesion and Chemotaxis in Multicellular Systems

E. Palsson; Biology, Simon Fraser University, Burnaby, BC, Canada

I will introduce a biologically realistic 3-dimensional mathematical model that facilitates visualizing the effect cell adhesion, stiffness, active force generation and chemotaxis have on the movement and signaling of cells in multicellular systems. The building blocks of the model are individual cells; each cell having certain given properties. The basic properties of a cell are: it is ellipsoidal and can deform under force, while conserving its volume, it adheres to other cells, and can generate active motive force. The response of a cell depends on its internal parameter state, and on the information it receives from its external environment, including neighbor cells, the extracellular matrix, and chemical signals. Since the model is based on known processes, the parameters can be estimated or measured experimentally. The cellular slime mold *Dictyostelium discoideum* is a widely used model system for studying a variety of basic processes in development, including cell-cell signaling, signal transduction, pattern formation and cell motility. Simulations of the cell movements during various stages of *Dictyostelium* development are shown and compared with experimental data. These include the chemotactic behavior of single cells, streaming during aggregation, and the collective motion of an aggregate of cells driven by a small group of pacemakers. The model predicts that the motion of two-dimensional slugs results from the same behavior that is exhibited by individual cells; it is not necessary to invoke different mechanisms or behaviors. I will also demonstrate how differences in adhesion between pre-stalk and pre-spore cells, affect the sorting and separation of those cell types, that occurs during the slug stage. I suggest and explain why chemotaxis alone might not be sufficient to achieve complete sorting and how including cell type specific adhesion contributes to much better cell sorting.

## Organization and Regulation of the Extracellular Matrix (1164-1172)

1164

### Phylogenetic Analysis of the Tenascin Gene Family

R. P. Tucker,[1] R. Chiquet-Ehrismann,[2] J. C. Adams[3]; [1]Cell Biology and Human Anatomy, University of California at Davis, Davis, CA, [2]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland, [3]Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

Tenascins are extracellular matrix glycoproteins that influence cell adhesion and motility during development and disease. In order to learn more about their origins and relationships to each other we have compared the tenascins encoded in the genomes of the pufferfish *Tetraodon nigroviridis* and *Takifugu rubripes* with those of tetrapods. Unlike amphibians, birds and mammals, which have four tenascin genes, the pufferfish each have five tenascins, including two tenascin-C paralogs: tenascin-CA and tenascin-CB. The predicted tenascin-R protein is identical in repeat and domain organization in each vertebrate class. All tenascin-W genes examined from pufferfish to man are found immediately adjacent to and on the opposite strand from the tenascin-R gene. The predicted tenascin-Ws encode variable numbers of fibronectin type III domains. This variation comes from duplications of the third fibronectin type III domain: *Tetraodon* has one, *Takifugu* two, chicken three, human six and mouse nine. Both pufferfish have tenascin-X genes found between the same genes that flank or overlap tenascin-X in birds and mammals. The predicted tenascin-X proteins contain nine (*Tetraodon*) or 20 (*Takifugu*) unique repeats (GKEQKKXTEGXNTLSP) between a single EGF-like repeat and three fibronectin type III domains. Phylogenetic trees confirm that there are four conserved members of the tenascin gene family: tenascin-R, tenascin-C, tenascin-W and tenascin-X. Tenascin-C and tenascin-R are the most closely-related, and may be derived from an ancestral gene duplication that also gave rise to tenascin-W. Tenascin-Xs are the most diverse group and the most distantly-related to other members of the family.

1165

### Studies on the Improvement of Skin Structure, Cell Adhesion, Differentiation, and ECM Synthesis by a Collagen-Like Peptide

N. Domloge,[1] C. Dal Farra,[1] E. Bauza,[1] A. Perrin,[1] J. Sabatier[2]; [1]Vincience, Sophia antipolis, France, [2]Faculté de médecine nord, CNRS, Marseille, France

Collagen plays a crucial role in tissue remodeling, in wound healing, and in maintaining the structural integrity of the skin. Being interested in the beneficial effects of collagen, we developed a synthetic oligo collagen-like peptide and studied its effect on cultured human skin cells and *ex vivo* models. Cell adhesion assay using HaCaT cells showed that when the cells were seeded in the presence of 10-6 M of the peptide, their adhesion to the microplates was clearly enhanced. Moreover, no evidence of increased cell migration caused by the peptide was observed after the cell migration assay. The peptide's role in enhancing cell adhesion was confirmed by time course studies and H&E staining of cultured fibroblasts. These studies revealed a nice uniform cell sheet in treated wells. Immunofluorescence studies on subconfluent fibroblasts showed that treatment of the cells with 10-6 M of the peptide increased the expression of ECM molecules (such as fibronectin, collagen I and III). Enhanced synthesis of keratin and adhesion molecules such as integrins (alpha 6, beta 1) and laminin 5, was observed in peptide-treated A431 cells. Moreover, *ex vivo* studies showed that human skin samples treated with the peptide, exhibited a remarkably better epidermal and dermal structure than control samples. In the peptide-treated samples, no signs of stress were seen in the epidermis. Interestingly, immunofluorscence studies of the expression of differentiation markers in skin samples, such as filaggrin, showed an increased expression of filaggrin in peptide-treated samples. These studies show that the peptide can be of great use in the area of tissue regeneration and in topical skin care products. The results reveal that the new collagen-like peptide stimulates ECM biosynthesis and offers a large number of beneficial collagen-like effects.

1166

### Three Dimensional Culture Regulates Raf-1 Expression to Modulate Fibronectin Matrix Assembly

B. S. Winters, B. K. Mohan Raj, E. E. Robinson, R. A. Foty, S. A. Corbett; Surgery, Robert Wood Johnson Medical School, New Brunswick, NJ

HT-1080 human fibrosarcoma cells possess one activated allele of N-*ras* and lack the ability to assemble a fibronectin (FN) matrix when grown in two-dimensional (2D) culture. Recently, we showed that culturing HT-1080 cells as three-dimensional (3D) aggregates restored FN matrix assembly to these cells. We hypothesized that the culture environment may influence matrix assembly by altering the expression of Ras signaling

intermediates, such as Raf-1. We show that HT-1080 cells grown as 3D aggregates down-regulate Raf-1 protein expression when compared to cells grown in monolayer culture. Diminished Raf-1 protein expression in 3D is associated with reduced Raf-1 mRNA levels as determined by quantitative RT-PCR and not proteasome-mediated degradation of endogenous Raf-1. Interestingly, transient expression of a Raf-1 promoter-reporter construct demonstrates increased Raf-1 promoter activity in 3D when compared to 2D culture, suggesting that the transition to 3D culture may modulate Raf-1 mRNA stability. Finally, to confirm that altered Raf-1 expression correlates with the increase in FNA matrix assembly, we used siRNA knockdown of Raf-1. This restored the ability of HT-1080 cells in 2D culture to assemble a FN matrix. Moreover, over-expression of Raf-1 prevented FN matrix assembly by HT-1080 cells cultured in 3D and resulted in decreased aggregate compaction. This work provides new insight into how Raf-1 expression may be regulated, allowing the identification of new molecular targets that could rescue the transformed phenotype.

1167

**Integrin β4 Regulates Migration Behavior of Keratinocytes by Determining Laminin-5 Matrix Organization**

B. U. Sehgal,[1] S. Matzno,[1] P. J. DeBiase,[1] T. Chew,[1] S. B. Hopkinson,[1] A. Russell,[2] M. P. Marinkovich,[3] J. C. R. Jones[1]; [1]Cell and Molecular Biology, Northwestern University, Chicago, IL, [2]Molecular and Cell Biology, Cytokinetics, Inc., South San Francisco, CA, [3]Program in Epithelial Biology, Stanford University, Stanford, CA

In hemidesmosomes, α6β4 integrin mediates stable matrix adhesion. Whether α6β4 integrin also regulates migration remains controversial. Here, we analyzed β4 integrin-deficient keratinocyte motility in vitro. Intriguingly, β4 integrin-deficient cells lack a polarized actin cytoskeleton, exhibit reduced levels of activated cofilin, and move in circles, a behavior that mirrors the circular arrays of laminin-5 in their matrix. Upon expression of wild-type β4 integrin, rescued cells show polarized actin and activated cofilin, lay down laminin-5 tracks, and exhibit directed migration, the behavior of wild-type keratinocytes. The subunits of laminin-5 in the matrices of both β4 integrin-deficient and wild-type cells appear identical, which suggests that laminin-5 organization regulates motility. In support of this, directed migration of β4 integrin-deficient keratinocytes is restored when they are plated on the laminin-5-rich matrix of wild-type keratinocytes, whereas wild-type keratinocytes show circular migration on matrix of β4 integrin-deficient keratinocytes. Laminin-5 antagonists inhibit these motility behaviors. To dissect the mechanism via which β4 integrin regulates laminin-5 matrix assembly and hence specifies motility behavior, we have investigated the role of the small GTPases RhoA, Rac1 and Cdc42. β4 integrin-deficient keratinocytes exhibit low Rac1 activity compared with their wild-type counterparts. Moreover, dominant negative Rac1 and Cdc42 both impact the motile behaviors of wild-type keratinocytes while dominant negative RhoA does not. However, only wild-type keratinocytes expressing dominant negative Rac1 exhibit the circular migration of β4 integrin-deficient cells. In summary, the migration behavior of keratinocytes is regulated by the precise organizational state of laminin-5 matrix. Directed migration of keratinocytes is determined by tracks of laminin-5 whose assembly is β4 integrin-dependent and involves remodeling of the actin cytoskeleton via a Rac1/cofilin signaling cascade.

1168

**Effects of Cell Traction Force Patterns and Focal Adhesion Distribution on Fibronectin Assembly**

C. A. Lemmon,[1] C. S. Chen,[2] L. H. Romer[3]; [1]Biomedical Engineering and Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, MD, [2]Bioengineering, University of Pennsylvania, Philadelphia, PA, [3]Anesthesiology and Critical Care Medicine, Cell Biology, and Pediatrics, Johns Hopkins University, Baltimore, MD

Extracellular matrix (ECM) proteins are assembled into a structured matrix by cell traction forces applied at focal adhesions. We have hypothesized that it is not only the magnitude but also the directionality and distribution of these traction forces which drive successful matrix assembly. We have previously demonstrated the use of microfabricated post array detectors (mPADs) to measure both cell traction forces and assembly of fibronectin. Here, we examine the effects of the distribution of focal adhesions and cell traction forces on fibronectin assembly. We have quantified the surface area of focal adhesions contiguous to the cell edge and focal adhesions located in the interior of the cell. We have also separated vectors of cell tension at the cell edge from tension vectors beneath the interior of the cell. Using these sorting techniques, we have examined the relationship between cell traction distribution and fibronectin fibrillogenesis. Our results indicate that cells with a higher percentage of interior traction forces assemble fibronectin into fibrils more efficiently. We have also investigated the role of Focal Adhesion Kinase (FAK) expression in the distribution of focal adhesions and cell traction forces. Using FAK-null and FAK-re-expressing mouse embryo fibroblasts, we have shown that the absence of FAK expression leads to a significant decrease in the percentage of adhesions in the interior of the cell as well as a significant decrease in fibronectin assembly. Thus we demonstrate that fibronectin assembly is dependent upon FAK-regulated modulations of focal adhesion distribution and cell traction force patterning.

1169

**Regulation of Integrin Activation and Fibronectin Fibrillogenesis by Galectin-3 Binding to Mgat5-modified N-glycans**

J. G. Goetz,[1] A. Lagana,[2] P. Cheung,[3] A. Raz,[4] J. W. Dennis,[3] I. R. Nabi[1]; [1]Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, [2]Pathology and Cell Biology, University of Montreal, Montreal, PQ, Canada, [3]Samuel Lunenfeld Research Institute, Toronto, ON, Canada, [4]Karmanos Cancer Institute, Detroit, MI

Oncogenic signalling stimulates the dynamic remodelling of actin microfilaments and substrate adhesions that is essential for cell spreading and motility. Transformation is also associated with the increased expression of β1,6GlcNAc-branched N-glycans, products of Golgi β1,6-acetylglucosaminyltransferase V (Mgat5) that represent the favored ligand for galectins. Fibronectin fibrillogenesis, fibronectin-dependent cell spreading and cell motility are deficient in Mgat5-/- mammary epithelial tumor cells and exogenous galectin-3 stimulates RGD-independent, PI3K-dependent fibronectin matrix remodelling. Using Latranculin-A, an inhibitor of actin polymerization, we found that galectin-3 mediated fibronectin fibrillogenesis was associated with increased actin microfilament turnover that occurred even in the presence of RGD. Treatment with both RGD and galectin-3 was associated with the reduction of a latrunculin resistant actin pool. Galectin-3 induced fibronectin fibrillogenesis was associated with the formation of β1-integrin positive fibrillar adhesions labeled with a conformation-dependent anti-alpha5 monoclonal antibody SNAKA51, which extend and elongate along actin stress fibers. Moreover, formation of β1-integrin positive fibrillar adhesions was also induced by treatment of Mgat5+/+ cells with an anti-galectin-3 antibody. Our results indicate that endogenous galectin-3 binding to Mgat5-modified N-glycans results in

β1-integrin clustering that increases the off-rate for RGD-dependent binding to FN and disrupts an RGD-stabilized F-actin pool within focal contacts. Galectin-3-stimulated recruitment of integrin to the galectin lattice regulates the translocation of fibrillar adhesion along actin stress fibers and fibronectin fibril stretching by increasing both integrin mobility and actin filament turnover. Supported by the CIHR.

1170

### Fibulin-5 is Expressed By RPE Cells and is Found in Drusen

E. C. Tolhurst,[1] S. T. Leu,[1] W. C. Wayne,[1] K. L. Wingerd,[1] J. Davis,[1] W. P. Schiemann,[2] D. O. Clegg[1]; [1]MCDB, University of California, Santa Barbara, CA, [2]National Jewish Medical Center, Denver, CO

Mutations in genes encoding fibulin-5 (Fib-5) have been associated with Age-Related Macular Degeneration (AMD), yet little is known about its distribution or function in the eye. Expression of Fib-5 mRNA and protein were assessed in human donor eyes with and without AMD, and in cultured ARPE-19 cells. mRNA levels were measured using real time RT-PCR, and protein was localized in sections using antibodies to unique peptides and imaging with a confocal microscope. Adhesion of ARPE-19 cells to recombinant Fib-5 was measured, and secretion of mutant Fib-5 proteins from 293-T cells was analyzed. In cultured ARPE-19 cells, and in RPE-choroid tissue samples, Fib-5 mRNA could be readily detected by quantitative RT-PCR. Individuals with AMD did not have significantly higher levels of Fib-5 mRNA in samples of RPE-choroid. In tissue sections, Fib-5 protein immunoreactivity was detected in Bruch's membrane, RPE, choroid, and some drusen. Recombinant wildtype Fib-5 supported adhesion of cultured ARPE-19 cells. Some mutations in Fib-5 associated with AMD altered their secretion properties when expressed in 293-T cells. These results show that Fib-5 is synthesized by RPE cells and deposited in Bruch's membrane and some drusen. Fib-5 may be a novel adhesion molecule for RPE cells, and mutations may alter secretion of Fib-5, which could contribute to AMD.

1171

### Detection of Extracellular Proteasome in the Human Alveolar Space

S. U. Sixt,[1] D. Spyrka,[1] J. Hakenbeck,[1] M. Beiderlinden,[1] H. P. Jennissen,[2] J. Peters[3]; [1]Klinik für Anästhesiologie und Intensivmedizin, Universitätsklinikum Essen, Essen, Germany, [2]Physiologische Chemie, Universitätsklinikum Essen, Essen, Germany, [3]Klinik für Anästheiologie und Intensivmedizin, Universitätsklinikum Essen, Essen, Germany

We tested the hypothesis that biologically active proteasome is present in the alveolar space of healthy humans and plays a role in the intraalveolar protein degradation. Methods: Following approval of the local ethic commitee and informed written consent, a bronchoalveolar lavage (BAL) was performed in 8 healthy humans during intravenous anesthesia. Proteasomal activity was measured using specific proteasomal fluorogenic substrates [BZ-VGR-AMC, Suc-LLVY-AMC and Suc-LLE-AMC] and $J^{125}$ albumin, with or without the specific proteasome inhibitor epoxomicin. Molecular weight of the BAL-supernatant were determined by gelfiltration and the revealed hydrolyzing activity at a molecular weight of 700 kDa were analyzed by ESI-QqTOF- mass spectrometry. Results: All proteasomal fluorogenic substrates were hydrolyzed by the supernatant and cell pellet of the BAL and were significantly inhibited by epoxomicin, with the major proteolytic activity detected in the supernatant. No correlation was found between the proteasomal hydrolysis in the BAL supernatant and LDH activity, the total cell count in cell pellet and the fraction of avital cells in cell pellet, ruling out an artifact by cell lysis. Gelfiltration revealed hydrolyzing activity in the supernatant (700 kDa) shows after analyzing by ESI-QqTOF- mass spectrometry proteasome subunit α 3 (PSMA3), PSMA4 and PSMA6. Incubation with $J^{125}$ albumin showed an ATP and ubiquitin independent albumin degradation, inhibited by epoxomicin with a high mean cleavage rate of 101.8 µg/ml x h lavage of $J^{125}$ albumin. Conclusion Thus a biologically active and albumin degrading extracellular proteasome is present in the human alveolar space suggesting that it may play a role in protein degradation and maintainance of a low oncotic pressure in the alveolar space.

1172

### The Characterization of Hyaluronidase in *C elegans*

A. R. Chatel, D. C. Merz; Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada

Hyaluronidases are endoglycosidases that degrade glycosaminoglycans (GAGs) such as chondroitin and hyaluronan. In mammals, at least six genes encoding hyaluronidases have been identified. An additional gene, meningioma expressing antigen 5 (MGEA5), is unrelated to hyaluronidase by sequence but degrades hyaluronan *in vitro*. Hyaluronidases regulate levels of GAGs in many biological processes such as wound healing, inflammation and cancer and are being used during *in vitro* fertilization, therapeutic drug injections and surgical procedures. The objective of this study is to determine the developmental roles and GAG substrate specificity of hyaluronidase and MGEA5 in the nematode *C.elegans.* Deletion mutations in the *C.elegans* genes have been generated. The mutant strain resulting from a deletion in the single hyaluronidase gene is *hya-1(dm12)* and in the putative MGEA5 is *mge-1(ok1207)*. *hya-1(dm12)* is a presumed null allele with a deletion spanning 848 bp and eliminating most of the catalytic glycoside hydrolase domain. *mge-1(ok1207)* is also a presumed null allele with an estimated 1700 bp deletion. *hya-1(dm12)* and *mge-1(ok1207)* animals are viable and grossly normal, although brood sizes of *hya-1(dm12)* are reduced relative to wild type, suggesting a role in germline development or fertilization. Enzyme activity assays performed *in vitro* with total protein extracts prepared from wild type and mutant strains showed no detectable hyaluronan-degrading activity *in vitro.* However, similar enzyme assays performed with chondroitin as a substrate showed degradation by wild type and *hya-1(dm12)* mutant strains, with the *hya-1(dm12)* mutant exhibiting greatly reduced levels of degradation. Chondroitin, the most abundant GAG in *C.elegans,* is essential for reproduction and morphogenesis. Our results suggest that HYA-1 has a possible role in the germline and that its substrate in *C.elegans* is chondroitin. Double mutant strains are being generated to further examine genetic interactions and GAG substrate specificities.

## Membrane Receptors (1173-1197)

1173

### Defective TNF-receptor 1 Signalling and NFκB Activation in TRAPS Patients

B. S. Nedjai,[1] G. A. Hitman,[1] M. F. McDermott,[2] A. Ranki,[3] S. Stjernberg-Salmela,[3] R. J. Coughlan,[4] K. Minden,[5] P. Arkwright,[6] S. McKee,[7] Y. Chernajovsky,[8] M. D. Turner[1]; [1]Centre for Diabetes and Metabolic Medicine, Bart`s and the Royal London, Queen Mary's School of Medicine and Dentistry, London, United Kingdom, [2]Clinical Science Building, St James Hospital, Leeds, United Kingdom, [3]Department of Dermatology and

Venereal Diseases, Helsinki University Central Hospital, Helsinki, Finland, [4]Department of Medicine, National University of Ireland, Galway, Ireland, [5]Rheuma-Forschungszentrum, Deutsches Rheuma-Forschungszentrum Berlin, Berlin, Germany, [6]Academic Unit of Child Health, St Mary's Hospital, Manchester, United Kingdom, [7]Department of Medical Genetics, Belfast City Hospital, Belfast, Ireland, [8]Bone and Joint Research Unit, Bart`s and the Royal London, Queen Mary's School of Medicine and Dentistry, London, United Kingdom

Objectives Tumour necrosis factor-alpha (TNF) is a key molecule in inflammatory disease, mediating its actions through two receptors, TNF receptor 1 and 2 (TNFR1 and TNFR2). Over 40 *TNFRSF1A* (TNFR1) mutations have been described which lead to the autoinflammatory disorder TNFR-associated periodic fever syndrome (TRAPS). We addressed whether *TNFRSF1A* variants cause defective TNFR1 signalling and NF-κB activation. Methods Nuclear and cytosolic fractions were generated from peripheral blood leukocytes (PBLs) from 7 TRAPS patients (*TNFRSF1A* C73R, T37I, R92Q, P46L, ΔD42, T50M, T50K mutations) and 7 healthy controls. Western blot and ELISA analysis were performed using antibodies against NF-κB subunits. Electromotility shift assay (EMSA) analysis was performed using a $^{32}$P-labelled NF-κB probe. Statistical significance was determined using T-test. Results The pattern of subcellular RelA (p65) NF-κB subunit localisation in both controls and the TNFR1 mutants, T37I, R92Q and ΔD42 was primarily cytosolic. However, T50K, P46L and T50M mutation led to a pattern of RelA expression that was equally partitioned between the nucleus and cytosol. More significantly, major differences were observed from C73R cells where RelA protein was localised almost entirely within the nucleus. Western blot using antibody against IκB-α also revealed complete localisation of this protein to the nuclear fraction of C73R cells. EMSA and ELISA analysis confirmed an elevated level of NF-κB in the nucleus of C73R cells ($p$ = 0.006 for p65; $p$ = 0.0001 for p50) relative to controls. Conclusions These findings suggest that there is inappropriate NF-κB activation in some TRAPS patients, particularly the non-conservative substitution of arginine for cysteine (C73R) in exon 3 of the *TNFRSF1A* gene which leads to a defect in NF-κB signalling. Hyperactivation of NF-κB may explain some of inflammatory processes which occur in TRAPS and could therefore open new routes for therapeutic intervention

1174

## The Role of Arrestins in CCR7 Internalization

M. A. Byers, P. Calloway, C. M. Vines; Micro., Molec. Genetics & Imm., University of Kansas Medical Center, Kansas City, KS

G protein-coupled receptors (GPCRs) are transmembrane proteins that span the cell membrane seven times and couple to heterotrimeric GTP-binding (G) proteins. Following receptor activation, a conformational change in the receptor leads to activation of the heterotrimeric G-proteins, receptor phosphorylation and arrestin binding. This arrestin binding to GPCRs prevents further association of the receptor with G-proteins and thereby, plays an important role in GPCR desensitization. Arrestins regulate receptor trafficking during both internalization and during receptor recycling, wherein arrestins serve as adapters between GPCRs and endocytic proteins such as clathrin and AP-2. The CCR7 GPCR, is a chemokine receptor that is capable of binding to two distinct ligands, CCL19 and CCL21. To examine the role of arrestins in trafficking of CCR7 following binding of either of its ligands, we expressed CCR7 in a mouse embryonic fibroblast cell line (MEF) that lacked endogenous arrestin 2 and arrestin 3 (arrestin-deficient). We compared CCR7 internalization and recycling kinetics in these cells to congenic wild type MEF cell lines following exposure to CCL19 or CCL21. We found that receptor internalization following binding to the CCL21 ligand is regulated by arrestins. In contrast, CCR7 internalization following binding to CCL19 is an arrestin independent process. These results suggest that CCR7 is internalized via distinct pathways in response to exposure to its two cognate ligands.

1175

## Cellular Localization of Neutral Amino Acids Transporter ASCT2, EGF Receptor (EGFR) and Growth Hormone Receptor (GHR) in Human Enterocytes

N. E. Avissar,[1] L. Toia,[1] S. J. Frank,[2] C. K. Ryan,[3] H. C. Sax[1]; [1]Surgery, University of Rochester, Rochester, NY, [2]Medicine, Cell Biology and Endocrinology, University of Alabama at Birmingham, Birmingham, AL, [3]Pathology, University of Rochester, Rochester, NY

Treatment with EGF plus GH of rabbits with short gut or of monolayers of human $C2_{BBe}1$ increases Gln transport. The response is variable depending on the route of administration, likely due to receptors location. EGFR in human intestine is a basolateral (Bl) protein while in cancer it is also apical (Ap). It has been suggested that human GHR and ASCT2 (Gln transporter) are Bl, while in rabbit intestine ASCT2 protein is Ap. We investigated the membranal location of ASCT2 and EGFR in human intestine, Caco-2 and its subclone, $C2_{BBe}1$. GHR location was evaluated in the cell lines only. Cells were grown on Transwells until tight junctions were formed. Membrane proteins were biotinylated on Bl or Ap side and were isolated from cell extracts by avidin precipitation. The isolated proteins were subjected to immunoblotting for ASCT2, GHR, EGFR and $Na^+/K^+$ ATPase (a control Bl protein). Immunohistochemistry was used to establish the location of ASCT2 and EGFR in human intestine. In both cell lines, ASCT2 was present on both membranes with higher levels on the Bl membrane. EGFR and GHR were present mainly on the Bl membrane; there were smaller amounts of EGFR on the Ap side. GHR staining was negligible on the Ap side. In the human ileum, EGFR was confined to the Bl membrane of the crypts, while ASCT2 was located on the brush border membrane (Ap) of the villi tip. We conclude that because there is a difference between the intestine and the cell lines in EGFR and ASCT2 membrane location, the cell lines cannot be used as an intestinal model for the side-specific regulation of ASCT2 transport by EGF. Funded by NIH 5 RO1 DK47989-10.

1176

## Degradation of Calcium-sensing Receptors by the E3 Ubiquitin Ligase Dorfin

Y. Huang,[1] G. E. Breitwieser,[2] J. Niwa,[3] G. Sobue[3]; [1]Biology, Syracuse University, Syracuse, NY, [2]Geisinger Clinic, Weis Center for Research, Danville, PA, [3]Neurology, Nagoya University Graduate School of Medicine, Nagoya, Japan

The calcium sensing receptor (CaR) is a G protein-coupled receptor which is critical for regulation of systemic calcium homeostasis. Little is known about the mechanisms regulating CaR synthesis and degradation. Yeast two-hybrid library screening identified an E3 ubiquitin ligase dorfin as a binding partner for the intracellular carboxyl terminus of CaR. Coimmunoprecipitation was used to confirm the interaction between CaR and dorfin in HEK293 cells. Ubiquitinated CaR was observed in the presence of proteasomal inhibitor MG132; mutation of intracellular lysine residues abolished ubiquitination of CaR. Coexpression of dorfin decreased the steady-state protein level of CaR and increased ubiquitination. A dominant negative fragment of dorfin had opposite effects on CaR steady-state protein levels and ubiquitination. AAA ATPase p97/valosin-containing protein (VCP) formed a complex with CaR and dorfin in HEK293 cells, suggesting that dorfin interacts with CaR at the endoplasmic reticulum

(ER). Treatment with tunicamycin, an inhibitor of N-linked glycosylation, induced the appearance of the unglycosylated 115-kD CaR form, which was further increased by MG132. Tunicamycin also increased ubiquitination of CaR in the presence of MG132, indicating that CaR is ubiquitinated at the ER. Two more mature forms of CaR (150- and 130-kD) were unaffected by tunicamycin or MG132, suggesting that only the unglycosylated CaR is degraded by the proteasome via the endoplasmic reticulum-associated degradation (ERAD) pathway. In conclusion, CaR and dorfin interact both in vitro and in vivo, and ubiquitination and degradation of CaR is regulated by dorfin via the ERAD pathway. Funding by NIH GM58578.

1177

## Secretin Receptor Oligomers Form Intracellularly during Biosynthesis through Receptor Core Domains

C. S. Lisenbee, L. J. Miller; Cancer Center and Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, AZ

Oligomerization of numerous G protein-coupled receptors has been documented, including the prototypic class B secretin receptor. Recently, we proposed that the observed dominant negative inhibition of secretin receptor function occurred via the direct interaction of wild type and misspliced receptors, the latter of which were found in gastrinomas and pancreatic cancer. In the current project, our goal was to explore the molecular mechanism of this interaction using bioluminescence (BRET) and fluorescence (FRET) resonance energy transfer and fluorescence microscopy with a variety of receptor constructs tagged with luciferase (Rlu) or cyan or yellow (YFP) fluorescent proteins. BRET signals comparable to those obtained from cells coexpressing Rlu- and YFP-tagged wild type receptors were observed for similarly-tagged secretin receptor constructs in which all or part of the N-terminal domain was deleted. As expected, neither of these constructs bound secretin, and only the partially truncated construct sorted to the plasma membrane. Tagged receptor constructs having alanine substitutions for either or both of the glycine residues of a -GxxxG- motif in transmembrane domain seven also produced BRET signals above background, as did receptors lacking the majority of the C-terminal domain, including that important for phosphorylation-mediated desensitization. Of interest, all of the receptor constructs were capable of associating with wild type secretin receptors to form heteromers. Furthermore, treatment of receptor-expressing cells with brefeldin A to inhibit trafficking of nascent receptors did not eliminate the BRET signals attributable to either homomeric or heteromeric interactions. Morphologic FRET experiments confirmed the expected subcellular localizations of receptor oligomers. Cumulatively, these findings suggest that the receptor core, and likely the transmembrane domains other than a -GxxxG- motif, are responsible for secretin receptor oligomerization, and that this can occur during biosynthesis and maturation of nascent secretin receptors.

1178

## Regulation of IgA-pIgR Transcytosis by EGFR-MEK-ERK Signaling Pathway in Epithelial Cells

T. Su,[1] F. Luton,[2,1] M. Vergés,[3,1] S. M. Ulrich,[4,5] K. Hansen,[6] A. L. Burlingame,[6] K. M. Shokat,[4] K. E. Mostov[1]; [1]Dept. of Anatomy and Biochemistry and Biophysics and Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA, [2]Institut de Pharmacologie Moleculaire et Cellulaire, CNRSUMR6097, 06560 Sophia-Antipolis, France, [3]Laboratory of Epithelial Cell Biology, Genomics and Pharmacoproteomics Program Centro de Investigación Príncipe Felipe, Valencia, Spain, [4]Dept. of Cellular and Molecular Pharmacology, University of California San Francisco, San Francisco, CA, [5]Chemistry Department, Ithaca College, Ithaca, NY, [6]Dept. of Pharmaceutical Chemistry, Mass Spectrometry Facility, University of California San Francisco, San Francisco, CA

Polymeric IgA (pIgA) transcytosis mediated by the polymeric immunoglobulin receptor (pIgR) is a highly regulated process in polarized epithelial cells and hepatocytes for immune defense against pathogens and allergens. Binding of pIgA to pIgR stimulates transcytosis of pIgA-pIgR suggesting that a signaling mechanism regulates this process. Indeed, binding of pIgA to pIgR leads to activation of PKCepsilon, production of inositol 1,4,5-trisphosphate (IP3), elevation of intracellular $Ca_i$ and a rapid tyrosine phosphorylation on several proteins in pIgR transfected MDCK cells. Recently we have found that c-Yes, a Src family member of non-receptor tyrosine kinases, controls pIgA-pIgR transcytosis in rodent liver. Furthermore, we have identified that epidermal growth factor receptor (EGFR) acts as a c-Yes substrate in rat liver endosomes by using a novel technique combined with mass spectrometry. Here, we report that intravenous injection of pIgA into rats induces EGFR phosphorylation in rat liver endosomes. Similarly, pIgA binding to pIgR in cells increases EGFR and extracellular signal-regulated protein kinase (ERK) phosphorylation. The interactions among EGFR, pIgR and c-Yes are found in liver endosomes and pIgR transfected MDCK cells by co-immunoprecipitations. The intracellular co-localization of EGFR, pIgR and c-Yes is also seen by confocal microscopy. Inhibition of EGFR and ERK phosphorylation by either PD153035, a EGFR inhibitor, or U0126, a MEK inhibitor, blocks the pIgA stimulated pIgR transcytosis. Our data indicate that the signalling pathway of EGFR-MEK-ERK is involved in the pIgA-pIgR transcytosis.

1179

## GRK 6 Regulate the Dopamine $D_1$-like Receptor-mediated Inhibition of $Cl^-/HCO_3^-$ Exchanger Activity in Rat Intestinal Epithelial IEC-6 Cells

S. Fraga,[1] P. A. Jose,[2] D. B. Mount,[3] P. Soares-da-Silva[1]; [1]Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal, [2]Department of Pediatrics, Georgetown University Medical Center, Washington, DC, [3]Renal Divisions, Boston VA Healthcare System and Brigham and Women's Hospital, Harvard Medical School, Boston, MA

The aim of the present study was to investigate the effect of dopamine $D_1$-like receptor stimulation on the $Cl^-/HCO_3^-$ activity in rat intestinal epithelial IEC-6 cells. IEC-6 cells are endowed with a $Cl^-/HCO_3^-$ exchanger. The pHi recovery after alkalinization imposed by the removal of $HCO_3^-$ from extracellular medium was found to be a chloride-dependent, DIDS-sensitive and niflumate-insensitive process. The presence of the SLC26A6 anion exchanger was detected by both RT-PCR and immunoblotting analysis. Activation of dopamine $D_1$-like receptors with SKF 38393 inhibited $Cl^-/HCO_3^-$ exchanger activity, this being antagonized by the $D_1$ selective antagonist SKF 83566. However, effects of SKF 38393 on the exchanger activity were maximal at 5 min of exposure to the agonist and rapidly diminished with no effect at 15 min, suggestive of agonist-induced desensitization of $D_1$-like receptors. Pretreatment of cells with heparin (1 μM), a non-selective inhibitor of G-protein coupled-receptor kinase (GRK), prevented the loss of effects upon $Cl^-/HCO_3^-$ activity after 15 min exposure to SKF 38393. Overnight treatment with the anti-GRK 6A and anti-GRK 6B, but not with the anti-GRK 4, antibodies prevented the loss of SKF 38393-mediated effects. Both PKA and PKC signaling pathways contribute to regulation of the $Cl^-/HCO_3^-$ activity mediated by dopamine $D_1$-like receptors. These findings suggest that SLC26A6 is at least one of

the anion exchanger's family members responsible for $Cl^-/HCO_3^-$ exchange in IEC-6 cells. Dopamine $D_1$ receptors in IEC-6 rapidly desensitize to $D_1$-like agonist stimulation and GRK 6, but not GRK 4, appear to be involved in agonist-mediated responsiveness and desensitization.

1180

**Transactivation of the EGF Receptor in Human Mammary Epithelial Cells by UTP and ATP Requires Ectodomain Shedding of EGF Receptor Ligands**

D. L. Ippolito; Fundamental sciences directorate, Pacific Northwest National Laboratory, Richland, WA

Transactivation of growth factor receptors by P2Y nucleoside receptors contributes to cell migration and proliferation. This study characterizes EGF (epidermal growth factor) receptor-induced MAPK (mitogen-activated protein kinase) activation and nuclear translocation following exogenous ATP (adenosine triphosphate) and UTP (uridine triphosphate) application in a human mammary epithelial cell line. Live-cell microscopy in cells co-expressing retrovirally-transduced GFP-MAPK and nuclear-targeted RFP responded to 100-500μM UTP or ATP by nuclear translocation of GFP-MAPK within 1-2 min, with GFP-MAPK assuming perinuclear localization approximately 5 min later. Lower UTP doses (1-10μM) resulted in GFP-MAPK translocation to the nucleus in approximately 4-6 min, with most of the GFP-MAPK remaining in the nucleus up to 15-30 min post-treatment. Nuclear MAPK translocation at all UTP doses was blocked by pre-treatment with mAb225, a monoclonal antibody specific for the ligand-binding domain of the EGF receptor, suggesting transactivation of the EGFR by P2Y receptor activation. Western blot analyses probing for phosphorylated MAPK confirmed rapid activation of MAPK within 1 min after treatment with 100μM UTP, while lower UTP doses (0.01-1μM) did not induce MAPK phosphorylation until 5 min post-treatment. Further, the time course for MAPK phosphorylation was inversely proportional to UTP dose. 100μM UTP induced a rapidly reversible MAPK phosphorylation which returned to baseline by 15 min, while the lowest UTP doses tested exhibited elevated MAPK phosphorylation at 5-30 min. Phosphorylation of MAPK by all doses of UTP was significantly reduced after pre-incubation with mAb225, batimistat or AG1517. Similar results were observed when cells were treated with exogenous ATP. Taken together, our data suggest that EGFR ligand shedding mediates EGFR transactivation following P2Y receptor stimulation by ATP or UTP.

1181

**Helicobacter Pylori Vacuolating Cytotoxin, Vaca-induced Cellular Vacuolation and P38 Activation is Dependent on Translocation of its Receptor RPTPbeta to Lipid Rafts**

T. Hirayama,[1] M. Nakayama,[1] A. Wada,[1] J. Hisatune,[1] E. Yamasaki,[1] Y. Nishi,[1] J. Moss[2]; [1]Dept. of Bacteriology, Nagasaki University, Nagasaki, Japan, [2]PCCMB, NHLBI, NIH, Bethesda, MD

*Helicobacter pylori* vacuolating cytotoxin, VacA, induces multiple effects on epithelial cells, e.g., pore formation, vacuolation, mitochondrial damage, apoptosis, p38 activation, eventually leading to gastric injury. We reported that VacA interacts with target cells by binding to two types of receptor-like protein tyrosine phosphatases (RPTPs), i.e., RPTPα and RPTPβ, resulting in toxin internalization and vacuolation. Translocation of VacA with RPTPβ to lipid rafts and VacA-induced cellular alteration including vacuolation and p38 phosphorylation in AZ-521 cells, which mainly express RPTPβ, were inhibited by treatment with methyl-β-cyclodextrin (MCD). These results suggest that translocation of VacA with RPTPβ to lipid rafts is critical for internalization of the toxin following by vacuolation, and effects on signal transduction as well as p38 activation. In AZ-521 cells, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), which blocks anion channels, reduced internalization of VacA and subsequent vacuolation, but neither translocation of VacA to lipid rafts nor VacA-induced activation of p38 was inhibited. Similar to MCD treatment, however, incubation with phosphatidylinositol-specific phospholipase C (PI-PLC) inhibited these VacA-induced events. Neither NPPB nor PI-PLC did not affected VacA binding to cells and interaction with RPTPβ. Thus, VacA localization with RPTPβ to lipid rafts is critical for its internalization and cytotoxicities, such cellular vacuolation and p38 activation.

1182

**The LOX-1 Scavenger Receptor Recognises Phosphatidylserine and Apoptotic Cells in a Calcium-Dependent Manner**

J. E. Murphy, J. H. Walker, S. Ponnambalam; Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a member of the scavenger receptor family (Murphy *et al.* (2005) *Atherosclerosis* In Press) that mediates vascular responses to pro-atherogenic oxidized low-density lipoprotein particles. The extracellular ligand-binding domain of LOX-1 is homologous to the natural killer cell C-type lectins that mediate calcium-independent recognition of MHC class I-related molecules. We find that human LOX-1 recognises a key cellular phospholipid, phosphatidylserine (PS), in a calcium-dependent manner both *in vitro* and *in vivo*. We have expressed a soluble, folded and glycosylated extracellular domain of human LOX-1 using a baculovirus expression system and investigated its ability to bind membrane ligands. Protein lipid overlay assays have revealed that this recombinant LOX-1 protein binds PS but not phosphatidylcholine, phosphatidylethanolamine or phosphatidylinositol. PS recognition by LOX-1 required physiological levels (>1 mM) of calcium ions, suggesting that native LOX-1 binds PS under extracellular conditions where millimolar calcium ion levels are present. Magnesium ions cannot substitute for calcium ions in LOX-1-mediated recognition of PS indicating that the divalent cation requirement is calcium-specific. Full-length LOX-1 protein was expressed in transfected human cells and was found to bind PS-containing apoptotic bodies. This binding was dependent on calcium ions and reduced to background levels by divalent cation chelation, LOX-1 blocking antibodies or PS-containing liposomes. The LOX-1 scavenger receptor is thus a calcium-dependent phospholipid-binding protein, indicating ligand recognition and calcium binding to the C-type lectin superfamily is more complex than predicted.

1183

**The Adaptor Protein RACK1 Associates with the Beta Isoform of the Thromboxane $A_2$ receptor**

A. Parent, G. Laroche, E. Hamelin, J. Parent; Rheumatology Division, University of Sherbrooke, Fleurimont, PQ, Canada

The thromboxane $A_2$ receptor is a G protein-coupled receptor which is implicated in the regulation of diverse pharmacological events such as platelet aggregation as well as constriction and proliferation of vascular and bronchiolar smooth muscle cells. This receptor is expressed as two alternatively spliced isoforms (TPα and TPβ) which share the first 328 amino acids. Our previous studies have shown that only TPβ, but not TPα, undergoes agonist-induced and constitutive internalization and that this specificity is conferred by the C-terminus of TPβ. In order to identify

putative proteins which are implicated in this specific regulation, we used the yeast two-hybrid system to screen for proteins that interact with the C-terminus of TPβ. One of the novel interactions identified occurs with the adaptor protein RACK1 (receptor of activated kinase 1). RACK1 is a WD-repeat protein which appears to serve as a scaffold for numerous proteins such as protein kinase C and Src kinase. The interaction between RACK1 and the C-terminus of TPβ was confirmed with purified proteins in vitro and co-immunoprecipitations in HEK293 cells. These experiments showed that RACK1 is constitutively associated with TPβ and that this association is not affected by stimulation with the TP agonist U46619. We also performed detailed mapping studies of the interaction sites of the C-terminus of TPβ and RACK1. The minimum site for RACK-1 binding was mapped to amino acids 328-337 of the TPβ C-terminus. On the other hand, the third and the seventh WD repeat domains of RACK1 were found to be involved in the interaction with TPβ. Taken together, our results suggest a novel interaction between a G protein-coupled receptor and the adaptor protein RACK1.

1184

**Interaction Between Rab3D and the Polymeric Immunoglobulin Receptor in Lacrimal Acinar Cells**

W. Zhang, G. Jerdeva, S. F. Hamm-Alvarez, C. T. Okamoto; Pharmaceutical Sciences, University of Southern California, Los Angeles, CA

Rab3D, a small molecular weight ras-like GTP-binding protein, is localized to secretory vesicles of various exocrine secretory cells, such as the acinar cells of the pancreas, parotid and lacrimal glands, and the chief cells of the stomach. Immunofluorescence studies indicated that rab3D, the secretory vesicle marker, and the polymeric immunoglobulin receptor (pIgR) are colocalized subjacent to the apical plasma membrane of lacrimal acinar cells. Therefore, we explored the interaction between rab3D and pIgR to determine the role of rab3D in the regulation of pIgR trafficking in primary cultured rabbit lacrimal acinar cells. Pull-down of pIgR from resting lacrimal acinar cell lysates with $(His)_6$-tagged wild-type rab3D (rab3DWT) showed that rab3DWT and dominant-negative rab3DT36N, a GDP-locked rab3D mutant, bind to pIgR; however, the constitutively-active rab3DQ81L, a GTP-locked rab3D mutant, does not bind to pIgR. Furthermore, when GTPγS or GTP is included in the pull-down assays with rab3DWT, binding of pIgR is significantly inhibited. The binding characteristics of pIgR to rab3D were reproduced when cell lysates of pIgR-tranfected MDCK cells were used in pull-down assays. Stimulation of lacrimal acinar cell secretion with 100 μM carbachol (CCh) for up to 2 hours prior to lysis, followed by incubation of lysates from stimulated acini with recombinant rab3DWT, resulted in a loss of pIgR binding to rab3DWT. A recovery of binding occurred after longer incubations of cells with CCh (4~24 hours). These data suggest that rab3D binds to pIgR when in the GDP-bound form, and GDP exchanging to GTP leads to the disruption of interaction between pIgR and rab3D. These changes may regulate pIgR trafficking in lacrimal acinar cells in a stimulation-dependent manner.

1185

**Structural Inhibition of Sema3A Effects by Synthetic Molecules**

L. Roth,[1] C. Nasarre,[1] S. Grosch,[1] D. Aunis,[1] P. Hubert,[2] D. Bagnard,[1] G. Crémel[1]; [1]U575, INSERM, Strasbourg, France, [2]CNRS, Marseille, France

Semaphorins are guidance molecules involved in neuronal connexions formation and in cellular migration. Among all known semaphorins, Sema3A is a secreted molecule whose particularity is to exert a functional duality for a unique cellular type. All studies performed highlighted that this functional diversity depends on the formation of a receptor complex. This receptor complex is composed of receptor units, namely neuropilins 1 and/or 2, and of transduction units, among them plexins. For a better comprehension of the receptor complex formation, we tried to disturb Sema3A signaling by preventing its receptor complex assembly. We synthesized a molecule A mimicking a specific neuropilin 1 domain. Interaction studies showed the high dimerization capacity of this domain. This capacity depends itself on the presence of small structural interaction motifs. We performed sucrose gradient analysis in presence or not of the semaphorin and showed that receptor complex evolution is highly dynamic. Molecule A induced complex disassembly. We next performed functional tests. In a collapsus assay, molecule A at 10-8M is able to specifically prevent the inhibitory effect induced by Sema3A on growth cone of cortical neurons. This effect has been confirmed on neuropilin 1/plexin A1 transfected COS-1 cells, whose shrinkage induced by Sema3A is totally inhibited by synthetic molecule A at 10-9M. We also synthesized an analog of molecule A (molecule B), carrying a mutation in neuropilin 1 interacting motifs. Molecule B is unable to reproduce these effects and shows molecule A specificity. As the dimerization motif found in neuropilin 1 is present in many other Sema3A coreceptors (neuropilin 2, L1-CAM, Vascular Endothelial Growth Factors), this type of synthetic molecule could allow in the future the understanding of the receptor complex formation and of its consequence in semaphorin signaling.

1186

**Antidepressants Bind to the Phencyclidine Locus in the Nicotinic Acetylcholine Receptor**

P. Bhumireddy, H. R. Arias; Pharmaceutical Sciences, Western University of Health Sciences, Pomona, CA

**ASCB DECEMBER 2005** **Objective:** To characterize the antidepressant (AD) binding sites in the resting and desensitized *Torpedo* AChRs. To demonstrate that ADs induce AChR desensitization. **Methods: (1)** [$^3$H]Doxepin equilibrium binding and Scathard-plot; **(2)** radioligand competition experiments using well known noncompetitive antagonists such as [$^3$H]TCP, [$^3$H]dizocilpine, and [$^{14}$C]amobarbital as well as Schild-type analyses; and **(3)** AD-induced [$^3$H]cytisine binding modulation. **Results:** These experiments yielded the following results: **(1)** there is one high-affinity binding site ([$^3$H]Doxepin $K_d$ = 2.7 ± 0.7 μM) for ADs in the *Torpedo* AChR; **(2)** the AD affinity for the [$^3$H]TCP or [$^3$H]dizocilpine locus in the desensitized state follows the sequence: imipramine (I) = amitriptyline (A) > fluoxetine (F) > doxepin (D) bupropion (B), whereas in the resting state it is: F = A > I > D = B; **(3)** Schild-type analyses suggest that ADs may sterically interact with both PCP and dizocilpine sites in the desensitized state; **(4)** the PCP locus partially overlaps the dizocilpine locus in the desensitized state; **(5)** I and F also inhibited [$^{14}$C]amobarbital binding in the resting state. However, the observed $K_d$s were higher than that for [$^3$H]TCP inhibition; and **(6)** [$^3$H]cytisine binding was enhanced by ADs when the AChR was in the resting but activatable state. However, this enhancement was not observed when the AChR was in the desensitized state. **Conclusions:** Considering these results we concluded that: **(a)** ADs bind to a site that overlaps both the PCP and dizocilpine loci in the desensitized state, as well as the PCP site in the resting state. **(b)** ADs, except bupropion, may inhibit the AChR by inducing the desensitization process; and **(c)** AD practically do not bind to the agonist sites.

1187

**TLR4 Is Expressed and Functions as LPS Receptor In Human Fetal Astrocytes**

S. J. Choi, H. S. Park, S. J. Park, K. Lee, S. Kim, J. Y. Park; Dept. Microbiology, Institute of Basic Medical Science, Yonsei University, Wonju College of Medicine, Wonju, Kangwondo, Republic of Korea

Although CNS is segregated from immune systems by BBB, bacterial challenges are able to induce inflammatory responses in the brain. There have been accumulating results that astrocytes actively participate in ongoing immune responses in the CNS. Toll like receptors have been known to participate in the recognition of microbial components such as LPS, peptidoglycan, and dsRNA. Until now, eleven TLR members have been identified in human. Among these receptors, TLR4 mediates LPS signaling along with CD14 and MD-2. To date, there have been conflicting results for the expression and its function of TLR4 in human astrocytes. In this study, we investigated the functional expression of TLR4 in human astrocytes. Using RT-PCR and Western blotting, TLR4 expression was demonstrated in unstimulated human astrocytes. To determine cellular responses induced by LPS, we used RT-PCR and ELISA for analyzing cytokine production and EMSA for NF-κB activation. The expression of TNF-α, IL-6, and IL-8 was increased by LPS treatment in serum containing condition, but not in serum free condition. NF-κB was activated by LPS treatment in serum containing media. The supplement of sCD14 molecules to the cultures restored the LPS effect. To determine whether TLR4 acts as LPS receptor, we used neutralizing anti-TLR4 antibody, HTA125. The cellular responses induced by LPS were abolished by HTA125 treatement. Taken together, we conclude that TLR4 is expressed in human astrocytes, allowing the cells to recognize G(-) bacterial infection.

1188

**Functional Expression of P2Y1 Receptor in Human Retinoblastoma**

D. Kim,[1] J. Park[2]; [1]Department of Basic Nursing Science, Keimyung Univ. College of Nursing, Daegu, Republic of Korea, [2]Institute for Nursing Science, Keimyung Univ., Daegu, Republic of Korea

Expression profiles of P2Y receptors vary according to the species and the cell types. It has been also known that P2Y2 receptor is the most prominent one among P2Y subfamilies in the many ocular tissues including retina. Therefore, we examined any changes in the subtypes of P2Y receptors along with malignant transformation in human retina. To achieve this goal, we used $Ca^{2+}$ imaging technique and Western blot analysis in WERI-Rb-1 cell, a human retinoblastoma cell. ATP (10 μM) elicited strong but transient increase in $[Ca^{2+}]i$ higher than 80% in the WERI-Rb-1 cells (n=46) in a concentration-dependent manner. This $[Ca^{2+}]i$ increase was well maintained by higher than 90.7±1.0 % after external $Ca^{2+}$-depletion (n=48). Phospholipase C inhibitor (U-73122, 1 μM) modestly suppressed the ATP-induced response eliciting 10.4±1.8% increase in $[Ca^{2+}]i$ (n=55), while its isoform (U-73343, 1 μM) had no suppression (n=69). $Ca^{2+}$-ATPase inhibitor (thapsigargin, 1 μM) also elicited a modest suppression on the ATP-induced response eliciting 8.1±0.9% increase in $[Ca^{2+}]i$ (n=52). And $IP_3$ receptor blocker (2-APB, 20 μM) suppressed the ATP-induced response by 92.5±1.3% (n=71). An order of potency of P2Y agonists to evoke $[Ca^{2+}]i$ transients was 2-MeATP>ATP>>UTP=α,β-meATP, which was compatible with the subclass of P2Y1 receptor. The $[Ca^{2+}]i$ transients by application of 2-MeATP and/or ATP was also greatly suppressed in the presence of $P2Y_1$ selective blocker (MRS 2179, 30 μM). $P2Y_1$ receptor protein was identified by using Western blot in WERI-Rb-1 cells. Taken together, P2Y1 receptor is mainly expressed in retinoblastoma cell, which activates $Ca^{2+}$ release from internal $Ca^{2+}$ storage sites via the PLC-mediated $IP_3$ pathway. P2Y1 receptor expressed in retinoblastoma may be worth as a novel target of therapeutic approach to this lethal cancer. The present research has been conducted by the Bisa Research Grant of Keimyung University in 2005.

1189

**Regulation of Dopamine D5 Receptor Signaling in Lipid Rafts in HEK-293 Cells**

P. Yu, J. R. T. Altea, Y. Luo, R. A. Felder, P. A. Jose; Pediatrics, Georgetown Medical Center, Washington, DC

The D1-like dopamine receptors play an important role in the regulation of renal function and blood pressure. D1-like receptors are composed of D1 and D5 receptors. We have recently reported that human D1 receptors (hD1Rs) expressed in HEK cells are regulated in the low density sucrose gradient lipid raft fractions that contain caveolin-2. We now show that the human D5 receptor (hD5R) signaling is also regulated in lipid rafts. The hD5Rs, heterologously expressed in HEK-293 cells, range in size from 33 to 250 kDa. The majority of the hD5Rs are located in the lipid raft membrane (LRM). The D1-like agonist, fenoldopam (5μM) increased the adenylyl cyclase activity by 2.6± 0.2 fold (n=4) in LRM and 1.7± 0.6 fold in non-LRMs. The cholesterol depleting reagent, methyl-β-cyclodextrin (β-CD), decreased the stimulatory effect of fenoldopam on cAMP accumulation by 26% relative to cells not treated with β-CD. The hD5Rs co-fractionated with lipid raft marker proteins, flotillin-1 and flotillin-2 in low density fractions. The D5Rs also co-immunoprecipitated with caveolin-2β, and co-localized with caveolin-2 by confocal microscopy. In addition, caveolin-2β co-immunoprecipitated with flotillins 1 and 2 in HEK-hD5 cells. The results indicate that the hD5Rs, like hD1Rs, are regulated to a greater extent in LRM than in non-LRM.

1190

**New Tools to Investigate Trail and Trail Receptors**

B. Bonnin,[1] E. Sergent,[1] M. O. Jauberteau,[2] O. Micheau,[3] J. M. Viart,[1] F. Bousquet,[1] J. Wijdenes[1]; [1]Diaclone, Besancon, France, [2]CNRS-UMR, Limoges, France, [3]Inserm U517, Dijon, France

Receptors of the TNF/NGF family have been shown to mediate a number of physiological functions including induction of apoptosis. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in many tumour cells but only rarely in normal cells. Five distinct receptors have been described for TRAIL: TRAIL-R1 (DR4), TRAIL-R2 (DR5, TRICK), TRAIL-R3 (TRID, DcR1), TRAIL-R4 (TRUNDD, DcR2) and Osteoprotegerin (OPG). Monoclonal antibodies (mAbs) were raised against the different molecules to study their role in apoptosis. MAbs were generated against TRAIL (B-S23, B-T24), TRAIL-R1 (B-N28, B-N36, B-T35), TRAIL-R2 (B-D37, B-K29, B-L27), TRAIL-R3 (B-D44, B-E45, B-H47, B-N29) or TRAIL R4 (B-L34, B-R27, B-T32). These mAbs were evaluated in terms of their ability to detect by Western blot, immuno-precipitation or by flow Cytometry TRAIL and TRAIL receptors. Moreover, the mAbs were tested in bioassays and ELISAs were developed to quantify the soluble TRAIL and TRAIL receptors. Of the anti-TRAIL mAbs B-S23 is an agonistic mAb while the B-T24 is an antagonistic mAb. Within the anti-TRAIL-R1 mAb family only B-N36 is an antagonistic mAb. Among the mAb panel against anti-TRAIL-R2 the B-D37 behaves as an agonistic mAb whereas B-K29 reacts as an antagonist mAb. One mAb against the TRAIL-R3, B-D44, has antagonistic

activity. Antibodies against TRAIL-R1, R2 and R4 were found to precipitate the receptor with or without the DISC while none of the mAbs against TRAIL-R3 could precipitate the antigen. Only the mAbs against TRAIL-R3 and R4 could be used in Western blotting. For all four soluble TRAIL receptors an ELISA was set up with a range of 30 - 1000 pg/ml.

1191

**Use of Complementary Peptides to Characterize Potential New Binding Sites for Thrombin and the Thrombin Peptide, TP508**

J. S. Bergmann,[1] G. M. Fuller,[2] D. H. Carney[1]; [1]Human Biological Chem & Genetics, Univ. Texas Medical Branch, Galveston, TX, [2]Dept. Cell Biology, Univ. Alabama, Birmingham, AL

The discovery that protein sequences encoded by complementary strands of nucleic acids can bind to one another is known as the molecular recognition theory. A number of laboratories have generated peptides with sequences derived from the complementary nucleic acid strand of the ligand and shown that the ligands bind the complementary peptides (CP). Antibodies to these CP have been used to identify specific receptors that have been difficult to purify. The 23 amino acid thrombin peptide, TP508, initiates a cascade of signals involved in tissue repair without the proteolytic activity required to activate previously cloned thrombin receptors, suggesting that this molecule activates a separate non-proteolytically activated thrombin receptor. To help identify the receptor molecule to which TP508 binds, we designed and synthesized peptides complementary to TP508 and regions within the molecule thought to interact with its receptor. Based on evidence that TP508 binds to a thrombin receptor, we chose to determine if thrombin could bind specifically to the CP adsorbed to ELISA plates. Specific thrombin binding was determined using biotinylated thrombin ([B]-thrombin) alone and with a 100-fold excess of unlabeled thrombin (define nonspecific binding). One of these peptides specifically binds [B]-thrombin while other sequences do not. In binding experiments in which increasing concentrations of [B]-thrombin are added, half maximal binding to this peptide was 4.8 ± 0.2 nM (n=2 ± SD). Interestingly, this value is similar to that reported for high affinity thrombin binding to fibroblasts. Addition of TP508 inhibits the specific binding of [B]-thrombin suggesting that the CP has a three-dimensional structure similar to the thrombin-TP508 receptor on cells. Antibodies to these peptides may therefore be useful in characterizing the thrombin binding site that is activated by TP508. (Supported by OrthoLogic Corp.)

1192

**Transmembrane Segment Peptides Can Disrupt Cholecystokinin Receptor Oligomerization Without Affecting Function**

K. G. Harikumar, M. Dong, Z. J. Cheng, D. I. Pinon, L. J. Miller; Cancer Center, Mayo Clinic, Scottsdale, AZ

Oligomerization of the G protein-coupled cholecystokinin (CCK) receptor has been demonstrated to exist, but its molecular basis and functional importance are not yet clear. Here, we have explored the ability of peptides representing transmembrane (TM) segments of this receptor to competitively disrupt oligomerization of wild type CCK receptor expressed in COS cells. Precedent for this approach comes from $\beta_2$-adrenergic receptor studies (Heber, JBC 1996). We utilized bioluminescence resonance energy transfer (BRET) to monitor oligomerization of CCK receptor constructs tagged at the carboxy terminus with *Renilla* luciferase or yellow fluorescent protein. Synthetic peptides representing TM I, II, V, VI and VII were utilized. Of these, only TM VI and VII peptides disrupted CCK receptor BRET. Control studies established that the $\beta_2$-adrenergic receptor TM VI peptide that had disrupted oligomerization of that receptor had no effect on CCK receptor BRET. Of interest, disruption of CCK receptor oligomerization had no effect on agonist binding or biological activity. To gain further insight into the face of CCK receptor TM VI that was most relevant for the disruption of receptor oligomerization, we also utilized analogous peptides that replaced the natural residues in positions 315, 319, and 323 (interhelical face) or in positions 317, 321, and 325 (external face) with Ala residues. The Ala317,321,325 peptide eliminated the disruptive effect on CCK receptor BRET, while the other mutant peptide behaved like wild type TM VI peptide. This suggests that the basis of CCK receptor oligomerization reflects the external face of the helical confluence, with particular importance of TM VI. This is most consistent with external contact dimerization in which established seven-helix bundles associate, rather than crossed-domain dimerization that has been previously suggested. Receptor mutagenesis experiments are currently in progress to further extend these observations.

1193

**Heterodimerization Alters the Functional Properties of G Protein-Coupled Receptors**

M. R. Tougas, K. K. Vincent, H. Radhakrishna, N. A. McCarty; School of Biology, Georgia Institute of Technology, Atlanta, GA

G protein-coupled receptors (GPCRs) play central roles in the responses of many cells to various chemical stimuli. Although GPCRs were formerly thought to exist only as monomers and homodimers, recent studies have shown that some may form heterodimers. The angiotensin II type I (ATR1) and bradykinin ($B_2$R1) receptors, and the $\beta_1$ and $\beta_2$ adrenergic receptors ($\beta_1$AR, $\beta_2$AR), are two receptor pairs that have been shown to heterodimerize through biochemical methods, although the functional consequences of heterodimerization in these receptors are not known. We used electrophysiological techniques to study whole-cell current in response to stimulation in *Xenopus* oocytes. Dose-response curves were obtained in oocytes expressing $\beta_1$AR or $\beta_2$AR, either alone or in combination, using the subtype-selective agonists dobutamine and terbutaline, respectively. The dose-response curve for terbutaline was right-shifted while the curve for dobutamine showed increase slope in cells expressing both receptors as compared to cells expressing only the cognate receptor. Cells co-expressing ATR1 and $B_2$R1 underwent heterologous desensitization upon exposure to either ligand, suggesting co-regulation upon heterodimerization. To determine whether heterodimerization also induced co-internalization of these receptors, we quantified surface receptor abundance as a function of ligand stimulation using a cell surface receptor ELISA. Kinetics of agonist-induced internalization were different when ATR1 and $B_2$R1 were co-expressed versus when expressed individually, supporting the notion that heterodimerization also altered receptor trafficking. Heterodimerization between the $G_\alpha$q-coupled $B_2$R1 and the $G_\alpha$s-coupled $\beta_2$AR altered both the internalization of $B_2$R1 and the agonist dose response of $\beta_2$ARs. Heterodimerization of $B_2$R1 and $\beta_2$AR also revealed a novel signaling cascade where activation of the $G_\alpha$q-coupled $B_2$R1 led to stimulation of $G_\alpha$s signaling through the heterodimerized $\beta_2$AR. Taken together, these data indicate that heterodimerization within and between different classes of GPCRs affects receptor function. Sponsor: PURA program at Georgia Tech

1194

**Monitoring the Assembly of Hetero-Trimeric G-Proteins and $GABA_B$ Receptor Heterodimers by Multiplex Resonance Energy Transfer Approaches**

B. Breton,[1,2] M. Lagacé,[1,2] M. Bouvier[1,2]; [1]Biochemistry, Montreal University, Montreal, PQ, Canada, [2]Groupe de Recherche Universitaire sur le Médicament, Montreal, PQ, Canada

Seven transmembrane receptors are now known to interact with their cognate heterotrimeric G protreins as dimers or even higher oligomeric complexes. A particularly convincing example is provided by the metabotropic $GABA_B$ receptor (GBR) that requires the formation of a heterodimer between GBR1 and GBR2 to constitue a functional receptor. GBR1 harbors the only hormone binding site but, in the absence of GBR2, is retained intracellularly. Its interaction with GBR2, allows the targeting of the heterodimer to the cell surface and assures its functional coupling to the G protein. However, neither the exact stoechiometry nor the structural organization of the complex have been studied in living cells. Here we multiplexed two bioluminescence resonance energy transfer techniques ($BRET^1$ and $BRET^2$) along with fluorescence resonance energy transfer (FRET) to probe the formation of GBR/G protein complex. Individually, neither GBR1 nor GBR2 could interact with any of the G-protein subunits tested ($G\alpha i1$,$G\beta 1$, $G\gamma 2$). However, a truncated version of GBR1 that can be trafficked to the plasma membrane in the absence of GBR2 was found to interact with the G-protein subunits indicating that plasma membrane trafficking permits GBR1 pre-coupling to the G-protein. This contrasts with the lack of constitutive association between GBR2 and the G-protein indicating a different propensity of the two receptor subtypes to spontaneously engage the G-protein. Upon co-expression of the two receptors, interaction between each of them and the G-protein subunits could be readily observed confirming the formation of a pentameric complex that could lead to G protein activation. In addition to provide new insight on the nature of the GBR/G protein complex formation, our study provides a proof of principle that RET multiplexing allows to monitor the formation of multimeric protein complexes in living cells.

1195

**Increased Insulin Signaling and Receptor Density in Calreticulin Deficient Cells**

S. Jalali, N. Mesaeli; Division of Stroke and Vascular Disease, Winnipeg, MB, Canada

Akt/PKB is one of the down stream signaling molecules of insulin receptor. Akt mediates many significant biological functions such as growth, survival and differentiation. Calreticulin is an endoplasmic reticulum resident calcium binding protein and chaperone. Recent studies show that calreticulin deficient cells (*crt-/-*) have a higher rate of cell proliferation and resistance to apoptosis. The aim of this study was to determine the change in the insulin signaling in the absence of calreticulin. Treatment of *crt-/-* cells with insulin resulted in more than 2 fold increase in the Akt kinase activity and a significant increase in both pAkt (S473) and pAkt (T308). Wortmannin and LY294002 inhibited insulin mediated activation of Akt, emphasizing a role for PI3 kinase activity. Furthermore, insulin treatment in the presence of AG1024 (IGF receptor blocker) did not alter Akt phosphorylation level. To determine if the observed changes in the phosphorylation was due to the changes in phosphatase activity we used fluorescent-based tyrosine phosphatase (PTP) and protein phosphatase 2A (PP2A) assays. There was no change in the PTP activity; however, PP2A was significantly higher in the *crt-/-* cells. Using western blot analysis we observed a significant increase in the insulin receptor β-subunit and phospho-GSK-3β level (up-stream and down-stream effectors of that Akt) in the *crt-/-* cells. Overall, our data show that insulin receptor signaling is up-regulated in the *crt-/-* cells which may partly account for the increased survival signal in the *crt-/-* cells. Furthermore, *crt-/-* cells show increased rate of serine/threonine phosphorylation and dephosphorylation.

1196

**Vasopressin Up-Regulates Immediate-Early Gene Expression by Two Different Epidermical Growth Factor Receptor (EGFR) Transactivation Pathways in A-10 Cells**

L. Q. Fuentes,[1] G. Perdomo,[1] C. E. Reyes,[1] S. Troncoso,[1] C. D. Figueroa,[2] C. B. Gonzalez[1]; [1]Physiology, Universidad Austral de Chile, Valdivia, Chile, [2]Histology and Pathology, Universidad Austral de Chile, Valdivia, Chile

Vasopressin induces mitogenic responses via transactivation of EGFR. We studied the effect of vasopressin stimulus on the expression of the growth-related immediate-early genes (IEG); c-Fos and Egr-1, which are downstream targets of EGFR. In A 10 cells, derived from rat vascular smooth muscle cells, one hour of vasopressin treatment caused maximal response in the expression of these genes. This effect was completely abrogated by both the PKC inhibitor GF109203X and the EGFR inhibitor AG 1478. The MEK inhibitor PD98059 only blocked the Egr-1 AVP-induced up-regulation. Two wide-spectrum metalloproteinase inhibitors greatly reduced only the c-Fos AVP-induced up-regulation. In contrast, the PP1 Src inhibitor significantly diminished only the Egr1 transcriptional activation. These results suggest that the AVP simultaneously induces the EGFR transactivation by two different pathways, one of which involves a metalloproteinase and the generation of EGF from the heparin-binding EGF-like growth factor and the other the activation of Src/PYK2. Furthermore, the transactivated EGFR up-regulates these two IEG by two divergent pathways one of which is MEK/MAPK-dependent. Supported by grant 103261 from FONDECYT and DIUACH.

1197

**Requirement of Transforming Growth Factor Beta Differs between Members of the Glial Cell Line-Derived Neurotrophic Factor Family**

H. Peterziel,[1] K. Unsicker,[1] K. Krieglstein[1,2]; [1]Neuroanatomy, University of Heidelberg, Heidelberg, Germany, [2]Department of Neuroanatomy,, DFG Center of Molecular Physiology of the Brain (CMPB), University of Göttingen, Göttingen, Germany

Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) constitute target-derived factors with important roles in the development and maintenance of different parasympathetic neuron populations including ciliary ganglion (CG) neurons. Recently, we have shown that survival and signaling mediated by GDNF in CG neurons essentially requires transforming growth factor β (TGFβ). We have provided evidence that TGFβ regulates the availability of the glycosyl phosphatidylinositol (GPI)-anchored GDNF receptor alpha 1 (GFRα1) by promoting the recruitment of the receptor to the plasma membrane. We report now that in addition to GDNF, NRTN, but not persephin or artemin, is able to promote survival of CG neurons. Interestingly, in contrast to GDNF, NRTN is not dependent on cooperation with TGFβ, but efficiently promotes neuronal survival and intracellular signaling in the absence of TGFβ. Additional treatment with TGFβ does not further increase the NRTN response. Both NRTN and GDNF exclusively bind and activate their cognate receptors, GFRα2 and GFRα1, respectively, as shown by the use of receptor-specific neutralizing antibodies. Immunocytochemical staining for the two receptors on the surface of CG neurons reveal that, in contrast to the effect on

GFRα1, TGFβ is not required for recruitment of GFRα2 to the plasma membrane. Moreover, disruption of TGFβ signaling does interfere with GDNF-, but not NRTN-mediated signaling and survival. We propose a model taking into account data from GFRα1 crystallization and ontogenetic development of the CG that may explain the differences in TGFβ-dependence of GDNF and NRTN.

## ER to Golgi Transport (1198-1213)

1198

### Regulation of Glutamate Transporter Trafficking from the ER to Golgi Complex

Y. Liu,[1] A. M. Ruggiero,[2] S. Vidensky,[1] J. D. Rothstein[2]; [1]Neurology, Johns Hopkins University, Baltimore, MD, [2]Neurology and Neuroscience, Johns Hopkins University, Baltimore, MD

Excitatory amino acid transporters (EAATs) are the primary regulators of extracellular glutamate concentrations in the central nervous system. Their dysfunction may contribute to several neurological diseases. In spite of their importance in synaptic actions, little is known concerning the regulation of their trafficking from the ER to the cell surface. Here we show that the glutamate uptake activity and cell surface distribution of the transporters are regulated by two integral ER membrane proteins, GTRAP3-18 and RTN2.GRTAP3-18, a mammalian Yip6b is an inducible protein and able to reduce transporter activity both in-vitro and in-vivo. RTN2 is a member of reticulon family. Both proteins associated with transporters directly. Over-expression of GTRAP3-18 results in the ER retention of the transporters, whereas transporters exit out of the ER and reach the cell surface efficiently when expressed alone or co-expressed with RTN2. Moreover, GTRAP3-18 and RTN2 interact with each other, suggesting a crosstalk of their regulation on ER retention and export. Our data indicate that interaction of transporters with ER proteins can affect their ER to Golgi trafficking and surface composition of transporters may be adjusted by controlling their export from the ER.

1199

### A Cargo-dependent Variable Coat Assembly Mechanism Controls Organelle Homeostasis

Y. Guo, A. D. Linstedt; Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

The size and integrity of endomembrane compartments depends on balanced membrane flux, but the mechanism that determines this balance is unknown. Here, we tested the hypothesis that Golgi proteins regulate COPII assembly at the endoplasmic reticulum (ER) thereby determining the extent of membrane input to the Golgi and Golgi size. This hypothesis predicts that COPII assembly level correlates with availability of Golgi proteins in the ER, and that increased availability of Golgi proteins in the ER, if sustained, causes an imbalance favoring membrane input to the Golgi and Golgi growth. In support of the first prediction, a marked transient increase in assembly of the COPII component sec13p at ER exit sites was observed to coincide with the emergence of Golgi proteins from the ER during synchronous assembly of the Golgi apparatus. Increased COPII assembly was even maintained at steady-state under conditions in which Golgi proteins rapidly cycle through the ER. Remarkably, and in support of the second prediction, overexpression of a single Golgi protein induced both increased COPII assembly and an overall increase in the size of the Golgi apparatus. Although several Golgi proteins contain binding sites for the sar1 GTPase, increased membrane recruitment required the sec23/24 complex in addition to sar1. The outer coat components sec13/31 were not required and increased assembly occurred in the absence of GTP hydrolysis. Altogether, these results indicate that COPII assembly provides a variable exit rate mechanism underlying homeostasis of the Golgi such that the level of COPII assembly is determined, in part, by direct interactions between Golgi proteins and the COPII coat complex.

1200

### Structural and Biochemical Characterization of the Self-assembled COPII Cage Mediating Cargo Export from the Endoplasmic Reticulum

C. Gurkan,[1] S. M. Stagg,[2,1] D. M. Fowler,[1,3] P. LaPointe,[1] C. S. Potter,[2,1] B. Carragher,[2,1] W. E. Balch[1,4]; [1]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]National Resource for Automated Molecular Microscopy, The Scripps Research Institute, La Jolla, CA, [3]Department of Chemistry, The Scripps Research Institute, La Jolla, CA, [4]The Institute for Childhood and Neglected Disease, The Scripps Research Institute, La Jolla, CA

Coat protein complex II (COPII) machinery comprising the small Sar1 GTPase and the cytosolic protein complexes Sec23/24 and Sec13/31 mediates cargo export from the endoplasmic reticulum (ER). While the current models suggest concerted polymerization of the Sec23/24 and Sec13/31 complexes to drive ER membrane budding, elucidation of the actual mechanism of COPII cage assembly and structure has so far been elusive. We have now established that Sec13/31 can self-assemble to form a cage-like particle with ~60 nm diameter independent of the Sec23/24 complex. The size of these Sec13/31 cages is consistent with that previously observed for COPII vesicles *in vitro* and *in vivo*. Using cryo-electron microscopy (cryo-EM) and single particle analysis, we have reconstructed the Sec13/31 cages at 30 Å resolution to reveal a novel cuboctahedron geometry that is distinct from the icosahedral clathrin lattices involved in endocytosis. In the cuboctahedral Sec13/31 lattice, the asymmetric unit corresponding to each edge is composed of the Sec13/31 heterotetramer. We now propose a revised model for COPII assembly in which Sec23/24 plays a non-structural role as a multivalent ligand localizing the self-assembly of the Sec13/31 cage to specialized regions of the ER for cargo export. This new model is expected to stimulate further experiments to elucidate the dynamics of Sec13/31 cage assembly/disassembly requisite for understanding ER cargo export.

1201

### Analyzing the Role of Sec16 in Transitional ER Organization in *Pichia pastoris*

E. A. Montegna, M. Esaki, P. L. Connerly, B. S. Glick; Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

In most organisms, COPII vesicles bud from specialized subdomains of the endoplasmic reticulum known as transitional ER (tER) sites. We have identified the peripheral membrane protein Sec16 as an important determinant of tER structure in the budding yeast *Pichia pastoris*. Sec16 had previously been shown to interact with several COPII components in *Saccharomyces cerevisiae* which lacks tER sites. We reasoned that comparing of the roles of Sec16 in these two yeasts will lead to a better understanding of the organizational properties of *P. pastoris* Sec16 (PpS16). In a simple model of tER organization, Sec16 could independently associate with the ER membrane, and then organize COPII components. To test this idea, we overexpressed PpS16 in both *P. pastoris* and *S. cerevisiae*. While the overexpressed PpS16 correctly localized to tER sites in *P. pastoris*,

PpS16 did not colocalize with COPII components in *S. cerevisiae*. Likewise, overexpressed *S. cerevisiae* Sec16 (ScS16) properly colocalized with COPII components in *S. cerevisiae*, but did not appear to associate with the ER in *P. pastoris*, suggesting that a species-specific interaction is important for proper localization of Sec16. We are now creating ScS16/PpS16 chimeric proteins to further test this hypothesis. Both PpS16 and ScS16 contain a large N-terminal unconserved region (NTU). We will use our chimeric proteins to test whether this region may be important for the organizational properties of PpS16. We are also overexpressing the NTU of both PpS16 and ScS16 in each yeast to determine the effects on COPII distribution. Additionally, preliminary evidence indicated PpS16NTU that may self-associate; we are testing this possibility by subjecting purified PpS16NTU and ScS16NTU to analytical ultracentrifugation. Ultimately, we hope to understand how the biochemical properties of Sec16 affect tER organization in diverse organisms.

1202

### A RNA-Protein Complex that is Required for Efficient TGF-α Secretion in *Drosophila*

J. E. Wilhelm, M. Buszczak, S. Sayles; Carnegie Institution of Washington, Baltimore, MD

Proper trafficking of proteins through the secretory pathway is critical for a number of signaling events that establish the primary body axes. It has become increasingly apparent that a number of signaling molecules require specialized chaperones or processing for proper transit through the secretory pathway. During *Drosophila* oogenesis, the dorsal-ventral axis is establishd by localized signaling between the oocyte and the follicle cells by *gurken* (*grk*), a TGF-α homolog. We have identified a novel gene, *trailer hitch*, that is required for proper secretion of Grk. Mutations in *trailer hitch* cause Grk protein to accumulate in large membrane vesicles that appear to be derived from the ER. Consistent with a role in ER-Golgi trafficking of Grk, we have found that Trailer Hitch localizes to regions that overlap and surround ER exit sites and that mutations in *trailer hitch* disrupt the normal organization of ER exit sites. Surprisingly, *trailer hitch* contains an atypical Sm domain and a FDF domain, suggesting that it regulates some aspect of mRNA metabolism. Biochemical purification of Trailer Hitch complexes has revealed that Trailer Hitch is part of a large RNA-protein complex that includes the RNA helicase, Me31B, and the eIF-4E binding protein, Cup. Me31B and Cup also colocalize with Trailer Hitch at ER exit sites, confirming the *in vivo* relevance of their biochemical association with Trailer Hitch. We propose that the normal secretion of Grk requires the local translation of proteins at ER exit sites and that *trailer hitch* is required for this process. Because *trailer hitch* is present in virtually all eukaryotes, these findings raise exciting new possibilities for how mRNA localization and translation could interface with the classical secretory pathway to promote efficient protein trafficking in the cell.

1203

### The Role of Sar1B in the Assembly of Chylomicrons

A. K. Townley,[1] B. Jones,[2] P. Williams,[2] P. D. Watson,[1] C. C. Shoulders,[2] D. J. Stephens[1]; [1]Biochemistry, University of Bristol, Bristol, United Kingdom, [2]Medical Research Council Clinical Sciences Centre, Hammersmith Hospital, London, United Kingdom

The transport of proteins from the ER to the Golgi apparatus requires the COPII protein complex. COPII is comprised of multiple subunits and its assembly on the ER membrane is initiated by GDP:GTP exchange on the small GTPase Sar1. Two isoforms of Sar1 are present in humans (A and B). Mutations in Sar1B cause chylomicron retention disease and Anderson's disease, both of which are characterised by defective assembly and/or secretion of chylomicrons. Notably there are also multiple isoforms of other COPII subunits (e.g. there are four human isoforms of Sec24). We are investigating the role of Sar1B in the assembly of the other subunits of the COPII complex and in secretion of chylomicrons. We show that Sar1A and Sar1B are functionally interchangeable for many aspects of COPII function but show specific differences in terms of the assembly and secretion of chylomicrons.

1204

### CAP-ER, a Late ER Compartment in Cartilage Cells for Aggrecan Trafficking and Quality Control

T. L. Chen, H. Li, B. M. Vertel; Cell Biology and Anatomy, Rosalind Franklin University of Medicine and Science, North Chicago, IL

We present a novel late ER compartment in chondrocytes that we name CAP-ER (Chondrocyte Aggrecan Precursor containing, late ER compartment), based on the relatively high concentration of aggrecan core protein observed within it. Link protein, which stabilizes aggrecan-hyaluronan associations in the cartilage ECM, is present in CAP-ER. In contrast, oligomerizing cartilage ECM molecules, such as types II and IX collagen and cartilage oligomeric matrix protein (COMP), are not detected in the compartment, but are prominent in broader regions of the rER. The membranes enclosing CAP-ER structures are ribophorin-negative, and smooth without associated ribosomes. The limited colocalization of ER chaperones BiP, GRP94 and calreticulin with aggrecan core protein in CAP-ER is a consistent observation. The association of ER exit sites (ERES) with CAP-ER and sensitivity of the compartment to brefeldin A suggest that one aspect of its function is as a source for aggrecan precursors trafficking to the Golgi complex. Dedifferentiation of the chondrocyte phenotype induced by treatment with retinoic acid or passage at low density is accompanied by a shift in the distribution of remaining aggrecan precursors to CAP-ER, suggesting a role for the compartment in the chondrocyte-specific, posttranslational regulation of aggrecan production. Expression studies, including live cell imaging of GFP aggrecan domain proteins, demonstrate that G1-containing versions enter the CAP-ER of chondrocytes but are not sequestered in corresponding ER structures in non-chondrocytic cells. We suggest that CAP-ER is a late ER compartment unique to chondrocytes that acts as a holding station or gateway for aggrecan precursors, which will either progress through the secretory pathway, be retained or be targeted for degradation. Chondrocytes, in producing high levels of aggrecan and modulating its production, utilize CAP-ER as a specialized posttranslational processing station for aggrecan precursors.

1205

### Reconstitution of ER-stress Induced ATF6 Budding *in vitro*

A. Schindler,[1,2] R. Schekman[1,2]; [1]Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [2]Howard Hughes Medical Institute, Berkeley, CA

ATF6 is an ER-localized transcription factor precursor protein that traffics to the Golgi complex during ER stress. In the Golgi membrane, ATF6 is cleaved by proteases into a soluble transcription factor that induces gene expression in the unfolded protein response. We have reconstituted the budding of ATF6 using crude ER microsomes isolated from mammalian cells. Treatment of the lysate with DTT, a potent ER-stress inducer,

caused budding of ATF6 precursor into vesicles. The packaging of other cargo molecules was less strongly stimulated by treatment of the lysate with a reducing agent. Other requirements such as nucleotide and cytosol, and characteristics such as inhibition by nonhydrolyzable GTP analogs and dominant negative forms of the GTP-binding protein, Sar1, were similar for ATF6 and other cargo proteins. Experiments to identify proteins that interact with ATF6 indicated that it exists as a high-molecular weight complex stabilized in part by disulfide bond(s).

1206

**Segregation of CFTR from Conventional Cargo Proteins in the ER-Golgi Intermediate Compartments**

A. Niibori,[1] T. Okiyoneda,[1] T. Kouno,[1] I. Wada,[2] M. S. Suico,[1] T. Shuto,[1] H. Kai[1]; [1]Molecular Medicine, Faculty of Pharmaceutical Sciences, Kumamoto, Japan, [2]Cell Science, Fukushima Medical Univ, Fukushima, Japan

Cystic Fibrosis (CF) is the most common lethal genetic disease in Caucasians, which is caused by the mutation of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutant of CFTR in CF patient (~90%) is Delta F508 CFTR that has a trafficking defect from the endoplasmic reticulum (ER). Since Delta F508 CFTR functions as a regulated $Cl^-$ channel, it is very important to overcome the trafficking defect from the ER for the CF treatment. However, the trafficking pathway of CFTR remains unclear although previous reports indicated that trafficking pathway of CFTR in the early secretory pathway is non-conventional. To examine the differences in the trafficking pathway of CFTR and conventional cargo proteins, we compared the trafficking pathway of CFTR and vesicular stomatitis virus G protein (VSV-G), which utilizes the conventional trafficking pathway. Both proteins were retained in the ER by brefeldin A, which inhibits the ER-Golgi transport, and their trafficking pathways from the ER were examined by immunocytochemical analysis using dual color imaging. Our results showed that the trafficking pathway of CFTR was different from that of VSV-G. The temperature-mediated trafficking block revealed that CFTR is segregated from VSV-G in the ER-Golgi intermediate compartments (ERGIC). Inhibition of the retrograde transport from cis-Golgi to the ER induced the accumulation of CFTR in cis-Golgi, indicating that CFTR is retrieved from cis-Golgi to the ER. Moreover, we also observed that CFTR was further segregated from VSV-G in the post-Golgi vesicles. These data emphasize that CFTR utilizes the non-conventional pathway.

1207

**Export to and from the ERGIC is Mediated by Two Distinct Motifs in the GABA Transporter1 C-terminus**

H. Farhan,[1] V. Reiterer,[1] V. M. Korkhov,[1] H. Hauri,[2] E. Sztul,[3] H. H. Sitte,[1] M. Freissmuth[1]; [1]Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Institute of Pharmacology, Vienna, Austria, [2]Department of Pharmacology and Neurobiology, Biozentrum, University of Basel, Basel, Switzerland, [3]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL

Export from the ER is mediated by COPII coated vesicles. In mammalian cells these vesicles have been shown to homotypically fuse and generate the intermediate compartment (ERGIC). The nature of this compartment and the mechanism of ERGIC-to-Golgi transport have been intensely depated. According to the maturation hypothesis, ERGIC membranes fuse and generate the cis-Golgi network. Here cargo is transported passively from ERGIC to the Golgi. According to the stationary compartment hypothesis, the ERGIC is a real compartment. In this model COPI components were suggested to control ERGIC-to-Golgi transport. We show that the transmembrane protein GABA transporter 1 (GAT1) recruits the COPII component Sec24D via a motif in its cytosolic C-terminus. In the absence of this interaction, GAT1 leaves the ER in a non-concentrative manner. We also identified the binding site in Sec24D. After exit from the ER, GAT1 is transported to the ERGIC. Here, its export is controlled by a distinct, trihydrophobic motif in the C-terminus. Mutation of this motif to serines (GAT1-SSS) leads to retention in the ERGIC. This is supported by the finding the GAT1-SSS colocalizes with the dominant negative Rab1a (Rab1a-N124I). In addition this compartment is discontinuous with the ER because: (i) using fluorescence recovery after Photobleaching (FRAP) we found that GAT1-SSS doesn't recover 90 sec after bleaching. (ii) GAT1-SSS is capable of leaving the ER (determined by an in vitro budding assay). We propose a two-step model for the ER-to-Golgi transport of GAT1. First COPII mediates cargo concentration and export to the ERGIC. Then a motif in the C-terminus controls export from the ERGIC. These data strongly support the notion that ERGIC-to-Golgi transport is controlled by motifs in the cargo molecule itself and thus support the stationary compartment hypothesis.

1208

**A Role of ArfGAP1 in ER to Golgi Transport of the GABA Transporter1**

V. Reiterer, M. Freissmuth, H. Farhan; Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Institute of Pharmacology, Vienna, Austria

We have found that the C-terminus of the GABA transporter1 (GAT1) interacts with the COPII component Sec24D. A mutant that is deficient in this interaction (GAT1-RL/AS) fails to be incorporated into vesicles (determined by an in vitro vesicle budding assay). Here we find that beside export in COPII coated vesicles, GAT1 also undergoes a concentration process in the ER after the COPII step. When we mutated a trihydrophobic motif in the C-terminus of GAT1 to serines (GAT1-SSS), this mutant was retained in the ERGIC as well as in vesicular tubular clusters (VTC) that were continuous with the ER. Formation of VTC was disrupted by co-expression of the dominant negative version of the Arf1 exchange factor GBF1. Rab1 has been shown to support COPI coat recruitment to the ER. In agreement with this, the dominant negative version, Rab1a-N124I, also inhibited VTC formation. These results point to a role of COPI in the concentration process. ArfGAP1 has been recently shown to mediate cargo concentration into COPI vesicles. The GST-tagged wild type GAT1 C-terminus (GST-wt) was able to pulldown ArfGAP1 from a cytosolic lysate. The C-terminus also interacted with purified ArfGAP1. While GST-SSS also interacted with ArfGAP1, GST-RL/AS failed to interact with ArfGAP1. Importantly, the level of beta-COP recruited by GST-SSS was 4-fold lower than that recruited by the wild type version. GST-RL/AS did not recruit any beta-COP. As the RL-motif mediates the interaction with Sec24D, we propose that coat exchange from COPII to COPI takes place on the same (RL-)motif. Additionally, we propose that the adjacent trihydrophobic motif stabilizes the interaction with coatomer which is recruited by ArfGAP1. As GAT1 is an anterograde cargo, our data show that ArfGAP1 is probably involved in ER-to-Golgi transport.

1209

**Role of EHD Proteins in Secretory Pathway**

J. J. Blume, M. Plomann; Centre of Biochemistry and CMMC, University Hospital of Cologne, Cologne, Germany

The EHD (Eps15 homology domain-containing) protein family consists of four members in mammals, which share a conserved modular

arrangement of an N-terminal potential GTPase domain (Pfam database), a non-conserved central linker region and a conserved C-terminal EH domain. All isoforms appear to participate in the regulation of the cargo transport via tubular structures in clathrin-mediated endocytosis as well as in a clathrin-independent pathway. Furthermore, EHD1 is essential for the release of transmembrane cargo proteins from the recycling endosome as dominant-negative mutations in the P-loop region or close to the EH domain of EHD1 significantly reduce their recycling to the plasma membrane. Purified recombinant EHD1 wildtype protein is able to hydrolyze GTP verifying that the EHD proteins represent a novel GTPase protein family. In addition, all family members bind to phospholipid-enriched liposomes with highest affinities for phosphoinositides in vivo predominantly found at Golgi-derived membranes. Furthermore, in case of EHD4 overexpression of an EH domain deletion mutant appears to abolish the correct targeting of the secretory pathway cargo molecules LDLR-GFP and GFP-GPI. Both are retained in EHD4deltaEH-positve compartments, which are resistent to Brefeldin A treatment and were identified as PDI-positive ER-derived compartments. These data suggest that EHD proteins are not only important for the regulation of endocytic processes but also participate in secretory trafficking events.

1210

**The Role of Mammalian GTRAP3-18 in the Early Secretory Pathway**

S. Maier,[1] V. M. Korkhov,[1] A. Ruggiero,[2] J. D. Rothstein,[3] H. H. Sitte,[1] H. Farhan[1]; [1]Center for Biomolecular Medicine and Pharmacology, Institute of Pharmacology, Vienna, Austria, [2]Department of Pharmacology, Vanderbilt University, Nashville, TN, [3]Departments of Neuroscience and Neurology, John Hopkins University School of Medicine, Baltimore, MD

GTRAP3-18 has originally been identified as a modulator of the neural glutamate-transporter excitatory amino acid carrier 1 (EAAC1). Co-localization studies using fluorescently labelled markers revealed its sub-cellular distribution to be predominantly in the endoplasmic reticulum (ER). Data from the literature showed that GTRAP3-18 leaves the ER and recycles back by means of its last four amino-acids (KARE). In contrast to these results we find that GTRAP3-18 is resident in the ER in an oligomeric form (determined by fluorescence resonance energy transfer). In addition, GTRAP3-18 failed to bind to Sec24, the COPII component required for cargo incorporation into COPII coated vesicles, supporting the notion that it is a resident protein. To determine the effect of GTRAP3-18 on ER-to-Golgi transport, we used the temperature-sensitive viral stomatitis virus glycoprotein (VSVG-tsO45). The rate of ER-to-Golgi transport was significantly slowed in the presence of GTRAP3-18. We next wanted to determine the mechanism by which GTRAP3-18 exerts its effect. As GTRAP3-18 is resident in the ER, we hypothesized that it might block cargo concentration in the ER. Concentration of EAAC1 was reduced by 50% in cells expressing GTRAP3-18. Recently, Rab1 has been shown to recruit COPI components to the ER and to mediate concentration of cargo into vesicular tubular clusters. Additionally, GTRAP3-18 shares conserved domains with the prenylated Rab acceptor proteins that regulate intracellular trafficking. Thus, we hypothesized that GTRAP3-18 might act by blocking the effect of Rab1. When we used cytosol that highly expressed Rab1 and added it to semi-intact cells expressing EAAC1 alone or together with GTRAP3-18, the effect of the latter on EAAC1 concentration was completely overcome. Therefore, we conclude that GTRAP3-18 slows the rate of ER-to-Golgi transport by blocking the action of Rab1 on the ER.

1211

**Regulation of the p115 Golgi Tethering Protein**

A. Satoh,[1] M. Beard,[2] J. Shorter,[3] G. Warren[1]; [1]Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Physiology and Cellular Biophysics, Columbia University, New York, NY, [3]Whitehead Institute, Cambridge, MA

The p115 tethering protein (Uso1p in yeast) is essential for ER to Golgi and early Golgi transport. It comprises three domains: an N-terminal globular head, a coiled-coil tail, and a short, acidic C-terminus. The acidic domain has been implicated in binding to GM130 and Giantin, and by so doing was thought to help tether COPI vesicles to cis Golgi membranes. The coiled-coil domain has been shown to aid in the subsequent assembly of SNARE complexes containing syntaxin 5. We now show that the coiled-coil tail also binds to the small GTPase, Rab1, and this binding is inhibited by the acidic domain. Furthermore, the binding of GM130 and Giantin to the acidic domain stimulates p115 binding to Rab1, suggesting that this process is regulated. The data lead to a model in which GM130 and Giantin regulate the recruitment of p115 to cis Golgi membranes by opening up a rab1 binding site that can then catalyze downstream SNARE assembly events.

1212

**The Discovery of Novel Factors that Regulate the Intracellular Trafficking of Chs3 Using Yeast Functional Genomics**

K. K. Y. Lam, E. Conibear; Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada

The spatial distribution of many proteins within a cell is altered by stimulus from the external environment however, the connection between signal transduction and vesicle transport is only partially understood. We use the protein *chitin synthase 3* (Chs3) in the yeast *Saccharomyces cerevisiae* to model the process of stimulus-driven trafficking, as components of vesicle machinery and trafficking pathways are well conserved from yeast to mammals. Cell stress induces the translocation of Chs3 from stable intracellular pools to the plasma membrane. We have carried out a fluorescence-based screen on the collection of ~5000 yeast gene knockout mutants to discover genes required for the cell-surface transport of Chs3 under conditions of stress. This high-throughput screen identified, as expected, all genes currently known to be required for Chs3 surface translocation, as well as components of the stress-activated cell integrity Pkc1-Slt2 protein kinase cascade. Numerous uncharacterized genes were also identified. Many of these belong to known functional clusters, including cell wall biosynthesis, signaling, and lipid metabolism. We have further characterized one gene of unknown function, *DUC1*, which was found to be required for endoplasmic reticulum (ER) export of Chs3. Our data suggest that Duc1 may be acting in cooperation with a known Chs3 ER exit factor, Chs7. Elucidating the fundamental mechanisms of Chs3 transport may contribute to our understanding stimulus-driven trafficking in higher cells.

1213

**Antagonistic Functions of Ypt6 and Ypt31/32 in Transport through the Golgi**

A. A. Tokarev, N. Segev; Biological Sciences, University of Illinois, Chicago, IL

The Ypt/Rab GTPase family regulates all intracellular transport steps in eukarytic cells. Transport and recycling through the yeast Golgi involves at least three of the eleven Ypt GTPases: Ypt6 and the Ypt31/32 functional pair. However, it is not clear if these GTPases have discrete or

overlapping functions. Here, we used live fluorescent microscopy and genetic analysis to address this question. We found that the two GTPases co-localize in the Golgi. Specifically, while Ypt31/32 localize mainly to the trans-Golgi and also endosomes, Ypt6 mostly resides in the cis-Golgi and not in endosomes. Over-expression and double mutant analyses reveal that the two GTPases affect cell growth and transport through the exocytic pathway in an antagonistic way. While Ypt6 is required for Golgi-to-ER retrograde transport, Ypt31/32 is required for anterograde transport. Lastly, protein recycling to the trans-Golgi in ypt31/32 and ypt6 mutant cells cannot be suppressed by the over-expression of the other Ypt. In conclusion, we show that in endosome-to-Golgi transport Ypt6 and Ypt31/32 GTPases have parallel but discrete functions, while in exocytic transport and cell viability their functions are opposite.

## Endocytosis II (1214-1232)

1214

### Yeast Clathrin is Important for Normal Cortical Actin Dynamics and Progression of Sla2p Containing Patches during the Early Stage of Endocytosis

T. M. Newpher,[1,2] R. P. Smith,[3] V. Lemmon,[3] S. K. Lemmon[1]; [1]Molecular and Cellular Pharmacology, University of Miami, Miller School of Medicine, Miami, FL, [2]Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH, [3]Miami Project to Cure Paralysis, University of Miami, Miller School of Medicine, Miami, FL

Clathrin is a major vesicle coat protein involved in receptor-mediated endocytosis. Previously we found clathrin on highly mobile internal *trans*-Golgi/endosomal structures and on smaller cortical patches in yeast. Total Internal Reflection Fluorescence Microscopy (TIRFM) showed cortical clathrin patches are likely endocytic sites, as clathrin is recruited prior to a burst of intensity of the actin patch/endocytic marker, Abp1. Clathrin is also present at the cortex with internalizing alpha-factor receptor, Ste2p, and with epsins Ent1/2p, AP180s and Sla2p. Here we show that the coiled-coil domain of Sla2p interacts directly with the N-terminal conserved sequence of clathrin LC, similar to what has been observed between mammalian LC and Hip1/R. TIRFM of Sla2p and clathrin shows that both proteins are early endocytic patch components that disappear at the onset of Abp1/actin recruitment; however, clathrin is recruited to patches prior to Sla2p. Deletion of the Sla2p coiled-coil domain ablates the interaction with clathrin LC and the lifespan of Sla2-Δcc-GFP in cortical patches doubles from 37s to 80s. Clathrin-deficient cells show a dramatic increase in Sla2-GFP lifespan at cortical patches, as well as aberrant actin comet tails, suggesting an important role for clathrin in endocytic patch organization/progression. To better define the importance of LC-Sla2 interactions, cells expressing LCs that no longer interact with Sla2p, but still bind clathrin HC, were generated. These cells still display normal growth, TGN sorting, and clathrin coated-vesicle formation. Therefore, Sla2p/LC interaction does not appear to be required for the TGN functions of yeast clathrin. Currently, we are investigating dynamics of the early and late stages of endocytosis in the Sla2p binding mutants, to determine the role of the LC/Sla2p interaction during endocytosis.

1215

### Sorting Nexin 9 Regulates Actin Polymerization Through Modulation of N-WASP Activity

D. Yarar, C. M. Waterman-Storer, S. L. Schmid; Cell Biology, The Scripps Research Institute, La Jolla, CA

We have previously established that F-actin dynamics are required for multiple steps of clathrin-coated vesicle formation. While numerous endocytic accessory proteins have been linked directly or indirectly to the actin cytoskeleton, the regulation of actin dynamics at sites of endocytosis is not understood. We have identified Sorting Nexin 9 (SNX9), a dynamin GTPase binding partner, as a regulator of actin dynamics in mammalian cells. We find that SNX9 binds directly to the neuronal Wiskott-Aldrich Syndrome protein (N-WASP) and enhances N-WASP/ Arp2/3-mediated actin nucleation. In contrast to other known SH3 domain-containing N-WASP activating proteins, SNX9 does not require its SH3 domain for this stimulatory activity, indicating that another region of SNX9 participates in activation of N-WASP/ Arp2/3 mediated nucleation. In living cells, GFP-SNX9 is transiently recruited to clathrin-coated pits late in endocytosis and localization at coated pits does not depend on an intact actin cytoskeleton. However, inhibition of actin assembly/ disassembly dynamics results in the accumulation of GFP-SNX9 at coated pits and loss of the transient association of GFP-SNX9 with coated pits. In addition, we find that RNAi-mediated depletion of SNX9 dramatically alters cellular morphology and actin architecture. Overall, these results suggest that SNX9 is a regulator of actin assembly in endocytosis and functions through the regulation of N-WASP.

1216

### Distinct Functions For Arp2/3 Activators During Endocytosis

Y. Sun, A. C. Martin, D. Drubin; Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Nucleation of actin polymerization by the Arp2/3 complex plays an essential but poorly understood role during endocytic internalization. The Arp2/3 complex requires the binding of an activator to fully stimulate its nucleation activity. A fundamental question is why multiple Arp2/3 complex activators are needed to carry out endocytic internalization. Here, we use the budding yeast, S. cerevisiae, to determine how four Arp2/3 complex activators, Las17p (yeast WASP), Myo3/5p (type I Myosin), Pan1p (yeast Eps15), and Abp1p, cooperate during endocytic internalization. Because Myo3/5p has not been directly shown to activate the Arp2/3 complex in budding yeast, we first purified full-length Myo5p and demonstrated its ability to activate the Arp2/3 complex in vitro in a Vrp1p (yeast WIP)-dependent manner. Myo5p/Vrp1p Arp2/3 activation activity was higher than Pan1p and Abp1p, but lower than Las17p. These differences in the efficiency of Arp2/3 activation and the fact that Arp2/3 activators have been shown to localize to endocytic sites at different times suggested that these activators play distinct roles during endocytosis. We investigated the function of each Arp2/3 activator by analyzing the phenotypes of complete gene deletions and targeted mutations that prevent Arp2/3 binding. We found that while Las17p is important for initiating actin assembly at endocytic sites, Myo5p is most important for actin endocytic internalization. Together, these results provide new insights into how multiple Arp2/3 activators function together during force-generating processes such as endocytic internalization.

1217

### Negative Regulation of the Yeast Eps15-like Protein, Pan1p, an Arp2/3 Activator, by Sla2p, Related to Mammalian Hip1R

J. Toshima, J. Y. Toshima, Y. Sun, M. C. Duncan, J. T. V. Cope, D. G. Drubin; Molecular and Cell Biology, University of California, Berkeley,

Berkeley, CA
Endocytosis is important for receptor internalization, nutrient uptake, antigen presentation, pathogen internalization, and plasma membrane homeostasis. In both yeast and mammals, endocytic internalization is accompanied by a transient burst of actin polymerization. Pan1p, the yeast homologue of the mammalian endocytic protein Eps15, is an essential protein that is involved in both the internalization step of endocytosis and the organization of the actin cytoskeleton. Pan1p is a 160 kDa protein consisting of two Eps15 homology (EH) domains at the N-terminus, a central coiled-coil region, and acidic and proline-rich regions at the C-terminus. Pan1p physiologically interacts with several endocytic proteins implicated in actin cytoskeleton and endocytic function. Previously our group reported that Pan1p binds and activates the Arp2/3 complex through its C-terminal acidic region, and that the activity is negatively regulated by phosphorylation by the kinase Prk1p. In this study, we identified Sla2p, the yeast Hip1R-related protein, as a novel binding partner of Pan1p. We found that Sla2p binds to the coil-coil region of Pan1p and inhibits its Arp2/3 activation activity. We also found that the central coiled-coil region of Sla2p is important for the binding and the inhibitory activities. Furthermore, pan1 mutant defective in Arp2/3 activation partially suppresses the growth defect observed in sla2 deletion cells at the non-permissive temperature, providing evidence for the biological relevance of the Pan1p-Sla2p interaction. These results suggest that Pan1p activity is regulated by phosphorylation by Prk1p and binding to Sla2p.

1218

### A Putative Role for Phosphoinositides in the Subcellular Localization and Function of the E3 Ubiquitin Ligase, Neuralized

L. C. Skwarek,[1,2] M. K. Garroni,[1] G. L. Boulianne[1,2]; [1]Developmental Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]Medical and Molecular Genetics, University of Toronto, Toronto, ON, Canada
Neuralized is a key regulator of the Notch signalling pathway which is required for the development of most tissues in all multi-cellular organisms. In *Drosophila*, the endocytosis of the Notch ligand Delta in the signalling cell is essential for the transmission of the signal to a neighbouring cell. Neuralized is an E3 ubiquitin ligase that is required for the endocytosis of Delta in a subset of Notch signalling contexts, and in the absence of Neuralized, signalling does not occur. The precise mechanism by which Neuralized initiates signal transmission through Delta endocytosis is unknown. Here we have examined the interaction between Neuralized and phosphoinositides and its potential role in Delta trafficking. Through *in vitro* binding assays we have determined that Neuralized binds a number of different phosphoinositides, particularly those that play a role in intracellular trafficking from the plasma membrane. We have mapped the region required for this interaction and show that mutation of this phosphoinositide binding motif mislocalizes the protein in S2 cell culture. In addition, we have studied the role of the mutated form of Neuralized *in vivo* using the GAL4/UAS system. Using flies expressing wild-type and mutant transgenic Neuralized we are currently assessing the functional consequences of disruption of phosphoinositide binding *in vivo* with the goal of understanding how this interaction is involved in Delta trafficking and signal initiation.

1219

### Functional Expression Cloning Identifies a MAP Kinase Phosphatase as Modulator of Dopamine Transporter Function

O. V. Mortensen,[1] B. M. Prasad,[2] M. B. Larsen,[1] S. G. Amara[1]; [1]Dept of Neurobiology, Univ of Pittsburgh, Pittsburgh, PA, [2]Dept of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA
The antidepressant sensitive monoamine transporters are the primary mechanism for clearance of their respective neurotransmitter from the extracellular space and pharmacological studies have established that the transporters serve a pivotal role in limiting monoamine-mediated neurotransmission and ultimately in determining behavior. We have found that modulation of transporter number by activating protein kinase C (PKC) and thereby stimulating trafficking of carriers to and from the cell surface is a process that seems to vary with the expression system used. To identify molecules responsible for these differences we have successfully used functional co-expression cloning in *Xenopus* oocytes. This led to the identification of a MAP kinase phosphatase, MKP3, as a modulator of PKC induced internalization of transporters and other membrane proteins. Surprisingly conventional MAP kinase families are not involved in the PMA-stimulated internalization, as MAP kinase inhibitors had no effect on internalization, nor did the activity state of different MAP kinases (ERK1/2, p38 and SAPK/JNK) correlate with the PMA-induced down regulation. These results suggest that MAP kinase phosphatases modulate a novel signaling transduction pathway involved in the regulation of clathrin mediated endocytosis. To identify other proteins involved in the trafficking of neurotransmitter transporters we are currently using substrate-trapping methods to isolate target substrates of MKP3.

1220

### Expression of Sorting Nexin-2 Enhances Lamellipodia Formation by the Neuronal Rac1 and RhoG Guanine Nucleotide Exchange Factor Kalirin-7

D. C. Prosser,[1,2] J. K. Ngsee[1,2]; [1]Ottawa Health Research Institute, Ottawa, ON, Canada, [2]Department of Neuroscience, University of Ottawa, Ottawa, ON, Canada
Sorting nexin 2 (SNX2) is a Phox-homology (PX) and Bin/Amphiphysin/Rvs (BAR) domain-containing protein involved in sorting of receptors within the endocytic pathway. Although previous studies have indicated that SNX2 plays a role in targeting of proteins to lysosomes, the precise cellular roles of SNX2 are not well understood. In order to identify SNX2-interacting proteins, we performed a yeast two-hybrid screen of a human brain cDNA library and identified Duo, a Rac1 and RhoG guanine nucleotide exchange factor (GEF), as a putative interactor. Binding of SNX2 to kalirin-7, the rat orthologue of Duo, was confirmed by co-precipitation from extracts of Chinese hamster ovary (CHO) cells co-expressing both proteins, and the C-terminal BAR domain of SNX2 was specifically required for binding to kalirin-7. Interestingly, CHO cells co-expressing SNX2 and kalirin-7 displayed a robust lamellipodia phenotype indicative of Rac1 or RhoG activation. Co-expression of SNX2 and kalirin-7 significantly increased the proportion of cells displaying lamellipodia compared to cells expressing kalirin-7 alone (89.7 ± 3.3% and 28.3 ± 1.7%, respectively) or SNX2 alone (0.7 ± 0.5%), and this phenotype was dependent on the GEF activity of kalirin-7. Truncation of SNX2 revealed that the PX and BAR domains are required for induction of lamellipodia, and mutations within these domains were able to inhibit lamellipodia formation compared to wild-type SNX2. Finally, co-expression of SNX2, kalirin-7 and dominant-negative forms of RhoG and Rac1 revealed that RhoG, but not Rac1, is required for SNX2-mediated induction of lamellipodia by kalirin-7. Overall, these results suggest a novel role for SNX2 in regulating actin dynamics by modulating the function of kalirin-7.

1221

**The Heterotrimeric Gq Protein Directly Interacts with Various ARF-GEFs**

E. Dupre, G. Laroche, J. Parent; Rheumatology Division, University of Sherbrooke, Fleurimont, PQ, Canada

G protein-coupled receptors are widely expressed seven transmembrane receptors with diverse pharmacological effects which are tightly regulated. In the present study, we report novel mechanistic aspects of their intracellular signaling by a direct molecular interaction between the heterotrimeric Gαq protein and various guanine nucleotide exchange factors (GEFs) for the small GTPases ARF1 and ARF6. The GEFs included in our study were cytohesin-1, GRP1, EFA6 and ARF-GEP$_{100}$. All of these GEFs co-immunoprecipitated with Gαq in experiments carried out in HEK293 cells, although the association between Gαq and EFA6 appeared to be the weakest. Interestingly, cytohesin-1 and its dominant negative mutant cytohesin-1(E157K) preferentially associated with a constitutively active mutant of Gαq compared to wild-type Gαq. Using GST-cytohesin-1 and Gαq proteins purified from bacterial and Sf9 cells respectively, we show that there is a direct interaction between these two proteins. We previously demonstrated that internalization of the G protein-coupled receptor for thromboxane $A_2$ required Gαq signaling through a phospholipase Cβ- and protein kinase C-independent mechanism. We then studied the role of cytohesin-1 in the internalization of this receptor. Noteworthy, over-expression of cytohesin-1 or cytohesin-1(E157K) respectively decreased and increased cell surface expression of the receptor in HEK293 cells. Our data also shows that co-expression of the cytohesin-1(E157K) mutant inhibited agonist-induced internalization of the thromboxane $A_2$ receptor by 40% after 60 min of stimulation in HEK293 cells. Together, our results demonstrate for the first time a direct interaction between Gαq and GEFs for ARF1 and ARF6. We also provide evidence that cytohesin-1 is positively involved in Gαq-mediated internalization of a G protein-coupled receptor.

1222

**The Arf6 GEF BRAG2 Regulates Cell Adhesion by Controlling Endocytosis of β1 Integrins**

J. L. Dunphy,[1] T. Lasell,[2] R. Moravec,[1] P. Melancon,[2] J. E. Casanova[1]; [1]Cell Biology, University of Virginia, Charlotesville, VA, [2]Cell Biology, University of Alberta, Edmonton, AB, Canada

Accumulating evidence indicates that Arf6 regulates post-endocytic trafficking of a subset of membrane proteins, including β1 integrins, that are thought to lack interactions with the clathrin-based endocytic machinery and are instead internalized by clathrin-independent mechanisms that remain poorly defined. In some cell types, these proteins enter an Arf6-positive endosomal compartment that is morphologically and functionally distinct from early endosomes containing cargoes internalized via the clathrin-dependent pathway. Importantly, dominant negative Arf6 mutants impair recycling of these proteins to the plasma membrane, suggesting that this process requires active Arf6. BRAG2 is a recently described guanine nucleotide exchange factor (GEF) with specificity for Arf6 in vitro. We have identified a second, longer isoform of BRAG2 (BRAG2b), which like BRAG2a is ubiquitously expressed. Both isoforms activate Arf6 in vivo, as determined using a pulldown assay for Arf6-GTP. To determine the role of BRAG2 in Arf6 function, we performed an RNAi analysis using β1 integrin trafficking as a readout. Knockdown of Arf6 itself led to a 30% reduction in surface expression of β1 integrin and impaired both attachment and spreading of cells on fibronectin. Surprisingly, knockdown of BRAG2a and BRAG2b resulted in a 41% increase in surface β1 and a corresponding enhancement of both attachment and spreading. This effect was specific for integrin, as surface levels of both transferrin receptor and EGF receptor remained unchanged, and was also specific for BRAG2, as knockdown of a different Arf6 GEF, ARNO, had no impact on adhesion. Together these results suggest that Arf6 regulates both integrin internalization and recycling and that BRAG2 selectively regulates the endocytic step.

1223

**Probing Arf Guanine Nucleotide Exchange Factor (GEF) Specificity with Chimeric Proteins**

L. A. Cohen, J. G. Donaldson; Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, MD

EFA6 and Arno are two Arf guanine nucleotide exchange factors (GEFs) which contain a sec7 domain that catalyzes nucleotide release from Arfs, allowing these proteins to cycle from an inactive GDP bound state to an active GTP bound conformation. How and whether this domain contributes towards specificity of the GEFs for particular Arf family members is not well understood. The EFA6 family of GEFs has been shown to be specific for Arf6, while the ARNO/cytohesin family of GEFs may act on both Arf1 and Arf6. To address whether the sec7 domain can determine specificity for particular Arf family members, and to address the significance of an exchange factor's specificity, we created chimeras replacing either the sec7 domain of ARNO with the sec7 domain of EFA6A (AEA) or replacing the sec7 domain of EFA6A with the sec7 domain of ARNO (EAE), and examined the function of these chimeras in cells. Co-expression of EFA6A and Arf6, but not Arf1, led to the formation of distinctive vacuolar structures, while co-expression of ARNO with either Arf6 or Arf1 did not. Co-expression of the AEA chimera with Arf6, but not Arf1, led to the accumulation, in some cells, of vacuoles similar to those formed in cells expressing EFA6A and Arf6. Interestingly, co-expression of the EAE chimera with either Arf6 or Arf1 led to an accumulation of these structures. These studies suggest that the sec7 domain itself dictates substrate specificity and that in some situations the backbone of the exchange factor, not the specificity of the exchange factor per se, is important in determining cellular outcome.

1224

**Src-kinase Expression Inhibits Compensatory Endocytosis During Early Sea Urchin Development**

J. F. Covian-Nares, S. S. Vogel; National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health (NIH), Rockville, MD

Tyrosine phosphorylation is an important regulatory mechanism for modulating the response of cells to external stimuli. In sea urchin embryos, Src family kinases (SFKs) regulate cell division (Hiriyanna et al., Exp. Cell Res. **216**: 21-29). In most cells, exocytotic activity is balanced by compensatory endocytosis to maintain cell surface area. Between the 8- and 32-cell stages of sea urchin development, endocytotic activity is down-regulated. This result in the net increase in embryonic cell surface area required for subsequent cell divisions (Frejtag et al., Dev Biol, 2003. **259**: 62-70). While the mechanism responsible for regulating this form of compensatory endocytosis is poorly understood, tyrosine-phosphatase inhibitors were found to block this form of endocytosis (unpublished results). The goal of the present study was to determine if over-expression of Src-kinase would also block compensatory endocytosis. We coinjected ***SpSK1*** (S*trongylocentrotus purpuratus* egg Src kinase) RNA (0.51 μg/μl), and fluorescent dextran (0.1 mg/ml, labeled with either tetramethylrhodamine or cascade blue) into single blastomeres of two-cell stage embryos of *L. variegatus* and compared subsequent cleavage patterns, and FM 1-43 uptake (4 μM), with that of their non-injected sister blastomeres (n=25).

Control embryos were injected with fluorescent dextrans only. As expected, SpSFK1 RNA injection inhibited cell division, while control blastomeres and uninjected sister blastomeres continued to divide. Two-Photon Microscopy revealed that SpSFK1 RNA injection arrested FM 1-43 uptake. Normal uptake was observed in non-injected sister blastomeres, and in control injected cells. These results suggest that the balance of tyrosine kinase and phosphatase activity during early development regulates compensatory endocytosis.

1225

**A Confocal Microscopy Analysis of the Effect of Tyrphostin-8, a GTPase Inhibitor, on Transferrin Intracellular Pathways in Caco-2 Cells**

F. Norouziyan, W. C. Shen; Pharmaceutical Science, USC, Los Angeles, CA

The extent of transferrin receptor (TfR)-mediated transcytosis in cultured epithelial cell monolayers is usually very low due to the efficient recycling of TfR to the originated surface. However, transcytosis of transferrin (Tf) has been detected in several epithelial cell lines including Caco-2 cells. In this report, the effect of the GTPase inhibitor tyrphostin-8 (AG10), which has been shown previously to increase the transcytosis of Insulin-Tf conjugate in Caco-2 cells, was used to investigate the intracellular processing of endocytosed FITC-Tf in order to identify compartments involved in TfR-mediated transcytosis. Caco-2 cells grown on transparent filter membranes were pulsed with FITC-Tf, bound to the apical or basal cell membranes, in the presence and absence of AG10 at 37°C for 30 min. The cells were then fixed and labeled with an antibody against Rab11. The images were collected from different fields of the prepared slides by using a confocal laser scanning microscope. When FITC-Tf was bound to the apical surface of Caco-2 monolayers, a high level of perinuclear colocalization between FITC-Tf and Rab11 was observed. However, no significant colocalization between FITC-Tf and Rab11 was observed in cell monolayers that were pulsed with FITC-Tf bound to the basal surface. On the other hand, AG10 increased the colocalization of FITC-Tf and Rab11 with both apical and basal internalization. These results suggest that apically endocytosed Tf may reach an endosomal comopartment containing Rab11, which is not readily accessible from the basal endocytosis of Tf. However, AG10 can increase the transport of Tf to a Rab11-containing endosomal compartment from both the apical and the basal surface, suggesting the involvement of this compartment in transcytosis and slow-phase recycling of Tf in Caco-2 cells. (Supported by NIH GM063647 and HL064365)

1226

**Intracellular Cholesterol Traffic in Macrophages upon Internalization of Aggregated LDL**

I. Grosheva,[1] F. R. Maxfield,[1] L. M. Pierini[2]; [1]Biochemistry, Weill Medical College, New York, NY, [2]Surgery, Weill Medical College, New York, NY

A critical event in the developing atherosclerotic lesion is accumulation of macrophages around sub-endothelial LDL deposits, which are typically large aggregates of LDL rather than monomeric LDL particles. Internalization and digestion of these lipoproteins by macrophages results in the transformation of macrophages into cholesterol overloaded “foam cells” that play a role in both early atherogenesis and late lesional events. In this study, the early events following contact between macrophages and aggregated LDL and the intracellular fate of LDL-derived free cholesterol were investigated. Two different model systems were used to study the interactions between macrophages and aggregated LDL: LDL-coated latex beads and LDL aggregates formed by vortexing of monomeric LDL. The use of LDL-coated latex beads allowed the quantification of free cholesterol specifically within the endocytic organelles containing the LDL-beads. Fluorescence imaging and digital image analysis of filipin-labeled samples was used to quantify the free cholesterol associated with LDL-bead-containing organelles. Using this approach, we determined the kinetics of free cholesterol association with the LDL-bead containing compartments both during and after LDL-bead internalization, and we evaluated the effects of various inhibitors on the trafficking of free cholesterol derived from the LDL-beads. These quantitative studies of cholesterol trafficking were conducted in parallel with EM visualization of phagosome ultrastructure, which revealed changes in LDL-containing compartments over time. The results obtained with LDL-beads were compared to those obtained with aggregates of LDL, and a model for the trafficking of free cholesterol derived from LDL aggregates is proposed. Our approaches to visualize and quantify cholesterol trafficking following LDL particle internalization will allow us to further elucidate the cellular processes involved in foam cell formation.

1227

**Pyrene-labeled Sphingomyelins in Human Fibroblasts Exhibit Chain-length Specific Differences in Degradation, Recycling, Detergent Resistance and Lateral Distribution**

M. A. Koivusalo; Anatomy, Institute of Biomedicine, Helsinki, Finland

Sphingomyelin is an important structural component of mammalian cell membranes and the precursor for messenger molecules in a number of signalling pathways. In human fibroblasts the chain length of the *N*-linked acyl residue varies from 16 to 24 carbons but the significance of this structural variety is largely unknown. To address the significance of the SM acyl chain diversity we have here studied the behaviour of pyrene-labeled sphingomyelins ($Pyr_nSM$) of different pyrenylacyl chain lengths in living cells. Short-chain $Pyr_4SM$ and long-chain $Pyr_{12}SM$ were introduced into human fibroblasts as we have previously established their domain partitioning in model membranes. We found that the uptake of both lipids was rapid and that they were internalised into early sorting endosomes at a similar rate as assessed by fluorescence microscopy. After longer times, however, a larger fraction of $Pyr_{12}SM$ than of $Pyr_4SM$ resided intracellularly and was degraded into ceramide by lysosomal acid sphingomyelinase. These data suggest that $Pyr_4SM$ recycled preferentially to the plasma membrane while $Pyr_{12}SM$ was targeted to the late endosomal pathway. $Pyr_{12}SM$ associated with detergent resistant membranes more avidly than $Pyr_4SM$, in accordance with data from model membranes. The lateral organisation of $Pyr_nSM$ as well as the corresponding ceramides was investigated by monitoring their excimer to monomer fluorescence ratio (E/M) by microscopy in living cells. The data suggest that the E/M ratio of the long-chain probes was more sensitive to changes in membrane order than that of short-chain probes, presumably reflecting the difference in the transversal localisation of their pyrene moieties.

1228

**Cholesterol Mobilization By MLN64 START Domain**

M. Hölttä-Vuori, A. Mutka, E. Ikonen; Anatomy, University of Helsinki, Institute of Biomedicine, Helsinki, Finland

MLN64 is a late endosomal protein harboring a cholesterol-binding START domain. Here, we show that depletion of MLN64 results in the dispersion of late endosomes to the cell periphery. Overexpression of wild-type MLN64 is capable of rescuing the endosome dispersion in MLN64-

depleted cells while START domain mutants of MLN64 are not, suggesting a functional connection between MLN64 START-mediated sterol transfer and late endosome dynamics. Overexpression of MLN64 and especially the START domain alone has been shown to increase steroid hormone production in mitochondria (Watari et al. 1997, Proc. Natl. Acad. Sci. U.S.A. 94:8462-7). We provide evidence that in addition, overexpression of MLN64 increases the transfer of cholesterol to the ER for esterification. This effect is more pronounced upon expression of the START domain alone. The increase in cholesterol esterification is observed also in cells defective in NPC1 protein or incubated with the hydrophobic amine U18666A. These results suggest that when present in high amounts, MLN64 START domain is capable of transferring cholesterol between cellular pools, even bypassing low basal levels of cholesterol esterification characteristic to cells harboring late endosomal/lysosomal cholesterol accumulation.

1229

**Differential Requirement of Sphingolipids for Various Clathrin-independent Endocytic Mechanisms**

Z. Cheng,[1] D. Sharma,[2] R. Singh,[1] E. Holicky,[1] K. Hanada,[3] D. Marks,[1] R. Pagano[1]; [1]Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, MN, [2]Photometrics, Inc., Tucson, AZ, [3]Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan

Sphingolipids (SLs) are plasma membrane constituents that are essential for membrane structure and organization. In the current study, the requirement of SLs for endocytosis was investigated. First, we showed that the uptake of cargo [lactosylceramide (LacCer), albumin, interleukin 2 receptors (IL-2R) and dextran] internalized by clathrin-independent endocytic mechanisms was inhibited by overall SL depletion in CHO cells using either a mutant cell line defective in SL biosynthesis or inhibitors of SL synthesis; however these treatments had no effect on clathrin-dependent endocytosis. Second, depletion of glycosphingolipids (GSLs), a subgroup of SLs, blocked caveolae-mediated endocytosis of LacCer, but had no effect on uptake of IL-2R or dextran, two markers internalized through non-caveolar mechanisms regulated respectively by RhoA and Cdc42. The inhibition of caveolar endocytosis by SL depletion could be overcome by acute treatment with GM3 ganglioside, but not by sphingomyelin. Additional experiments demonstrated that SL depletion inhibited the non-clathrin, non-caveolar endocytic mechanisms by inhibiting the targeting of RhoA and Cdc42 to the plasma membrane. This inhibition could be partially restored by incubating cells with sphingomyelin, but not GSLs or ceramide. These results provide the first evidence that SLs differentially regulate multiple mechanisms of clathrin-independent endocytosis. (Supported by USPHS Grant GM22942)

1230

**Participation of Different Dynamin2 Spliced Variants in Distinct Endocytic Processes**

H. Cao, J. Henley, M. Awoniyi, M. A. McNiven; Center for Basic Research in Digestive Diseases and Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN

The dynamin family of large GTPases has been implicated in a variety of different endocytic events, although its participation in the fluid phase endocytic process remains controversial. Multiple spliced-variants of the Dyn2 isoform have been identified in epithelial cells and predicted to participate in distinct membrane trafficking events. In this study, we test the role of Dyn2 in clathrin-mediated endocytosis (CME) as well as macropinocytosis and fluid-phase endocytosis in a variety of different mammalian epithelial cells. Inhibiting all forms of Dyn2 was first performed using microinjection of purified antibodies and siRNA knock-down of Dyn2. We find that microinjection of two different purified dynamin antibodies known to inhibit Dyn2 function, or siRNA knock-down of Dyn2, significantly reduces the internalization of transferrin in cells, as well as uptake of the fluid-phase marker dextran. In contrast, Dyn2 antibody-injected or Dyn2 knock-down epithelial cells, grown in high serum or treated with EGF to stimulate the macropinocytotic pathway, internalize substantial amounts of fluid dextran. Following these broad blocks of Dyn2 function, we tested if a specific Dyn2 variant form might selectively support fluid uptake in cells. Expression of four different Dyn2 variants (aa, ab, ba and bb) reveals distinct cytoplasmic localizations and, when mutated, differentially attenuate the internalization of dextran and transferrin. Interestingly, the wt Dyn2ba form accentuates fluid uptake by more than 20% compared to mock transfected cells, while the Dyn2baK44A GTPase mutant reduces dextran uptake by 60%. Other Dyn2 forms had little affect on fluid phase endocytosis. These findings suggest that Dyn2 function is required for the uptake of fluid dextran in a variety of epithelial cells by endocytic pathways distinct from the macropinocytotic process while implicating distinct spliced variants in specific endocytic functions.

1231

**Surface Downregulation of ErbB2 by Antibody Crosslinking Involves an Unusual Endocytic Pathway**

A. M. De Mazière,[1] C. D. Austin,[2] S. M. van Dijk,[1] J. Klumperman,[1] R. H. Scheller[2]; [1]Department of Cell Biology, University Medical Center Utrecht, Utrecht, The Netherlands, [2]Genentech Inc., South San Francisco, CA

ErbB2 is an oncogenic member of the EGFR family of receptor tyrosine kinases and is overexpressed in 20-30% of human breast cancers. ErbB2 heterodimerizes with EGFR to potentiate its mitogenic signaling, partly through diversion from EGF-induced surface receptor downregulation. Trastuzumab and pertuzumab are humanized monoclonal anti-ErbB2 antibodies that inhibit tumor cell growth in vitro and in vivo without downregulating surface ErbB2 (1). Our aim was next to establish the endocytic pathway involved in downregulation of ErbB2 by crosslinking. We find that antibody crosslinking of trastuzumab or pertuzumab triggers ErbB2 surface downregulation through both induced endocytosis and altered endosomal sorting. Immunogold labeling of ultrathin cryosections revealed clusters of crosslinked ErbB2 within unusual endocytic profiles, distinct from typical clathrin-coated pits or caveolae, after only a few minutes of incubation. These structures appeared as narrow, deep tubules or folds, occasionally with a clathrin-coated pit at the bottom. After longer incubations, similar tubules were clustered adjacent to and occasionally fused with early endosomal vacuoles, positive for Hrs and poor in LAMP-1. Ultimately, crosslinked complexes appeared to be sorted to internal vesicles of multivesicular bodies. Antibody uptake kinetics in cells expressing mutant forms of ErbB2 suggested that crosslinking-induced downregulation involves ErbB2 kinase-dependent acceleration of endocytosis as well as cytoplasmic domain-dependent, kinase-independent endosomal sorting. These results support the idea that ErbB2 has dormant trafficking determinants that mediate surface downregulation after antibody crosslinking through an unusual endocytic pathway. (1) Austin C.D. et al. (2004) Mol. Biol. Cell 15, 5268-5282.

1232

### Transferrin Regulates the Trafficking of Transferrin Receptor 2 in Hepatoma Cells

M. B. Johnson, C. A. Enns; Cell & Developmental Biology, Oregon Health & Science University, Portland, OR

Transferrin receptor 2 (TfR2) is a homologue of the classical transferrin receptor (TfR1). TfR2 is predominantly expressed in the liver. Like TfR1, TfR2 binds diferric transferrin ($Fe_2Tf$), a serum protein that delivers iron to cells, but with 30-fold lower affinity. Interestingly, mutation or knockout of TfR2 results in the iron overload disease hereditary hemochromatosis (HH), suggesting that TfR2 functions in iron homeostasis rather than iron delivery. We have previously shown that $Fe_2Tf$ regulates TfR2 protein stability at physiologically relevant concentrations. The finding suggests that trafficking of TfR2 may be regulated by its ligand and important for its function. We have studied the localization and trafficking of TfR2 in HepG2 hepatoma cells that endogenously express TfR2 and in Hep3B hepatoma cells stably transfected with TfR2. Western blot analysis shows that treatment of HepG2 cells with bafilomycin, an inhibitor of lysosomal degradation, increases TfR2 levels, indicating that TfR2 is degraded by the lysosome, whereas treatment with proteosomal inhibitors does not effect TfR2 levels. Confocal microscopy studies show that TfR2 partially colocalizes with EEA1 and TfR1 in early and recycling endosomes. Treatment with $Fe_2Tf$ increases the fraction of TfR2 present in recycling endosomes. In nonpermeabilized Hep3B/TfR2 cells, TfR2 is also visible at the plasma membrane. Differential immunoprecipitation of cell surface and intracellular TfR2 from Hep3B/TfR2 cells 48 hours after seeding indicates that TfR2 distributes equally between intracellular compartments and the cell surface. The mutation Y23A in the cytoplasmic domain of TfR2 increases the proportion of TfR2 localizing to the cell surface, suggesting that the YQRV sequence in the cytoplasmic domain may serve as an endocytic motif. These results indicate that TfR2 most likely internalizes through a tyrosine-based motif and that Tf stabilizes TfR2 by altering its trafficking in hepatoma cells.

## Protein Targeting to the Cell Surface (1233-1246)

1233

### Utilizing the Power of Yeast Genetics to Search for Proteins that Regulate the Intracellular Fate of Secretory IgM μs Heavy Chains

G. Nadel, M. Mayrose, O. Burdelova, S. Bar-Nun; Biochemistry, Tel-Aviv University, Tel-Aviv, Israel

The mechanisms that control the developmentally regulated secretion and degradation of secretory IgM are not yet fully understood. The C-terminus of μs heavy chain and its penultimate Cys575, designated μstpCys, functions in B-cells as a *cis*-acting retention signal (Sitia et al. 1990 *Cell* **60,** 781-790). This motif is also sufficient to confer retention and degradation onto several reporter proteins in lymphoid and non-lymphoid mammalian cells. However, *trans*-acting cellular components that contribute to μs retention remain elusive. The remarkable conservation of the secretory pathway from yeast to man intrigued us to ask whether this μstpCys motif could confer retention also onto yeast secretory proteins. Hence, the μstpCys and its mutant inactive form μstpSer were grafted onto the C-terminus of invertase and secretion of this chimeric protein from *Saccharomyces cerevisiae* was followed by its enzymatic activity, or directly from spheroplasts using immunoblotting assays. Yeast neither synthesize immunoglobulins nor μstpCys is found in the yeast genome. Nonetheless, μstpCys, but not μstpSer, conferred cellular retention onto invertase. Although the retained invertase-μstpCys was not degraded, it was dislocated back to the cytosol. The crucial involvement of the penultimate cysteine, together with the stimulating effect of β-mercaptoethanol exclusively on the secretion of invertase-μstpCys, focused us on ER thiol-oxidoreductases. In *eug1Δ* strain, lacking the human protein disulfide isomerase homolog Eug1p, the increased secretion of invertase-μstpCys matched the unaffected secretion of invertase-μstpSer. It indicates that Eug1p contributes to μstpCys-mediated retention, although its mode of action remains to be elucidated. We conclude that the molecular mechanisms underlying secretory IgM secretion can be studied in yeast, exploiting its well characterized genetics.

1234

### Sar1 Regulates the Intracellular Transport of Kv111 Early in the Secretory Pathway

B. P. Delisle, J. K. Slind, J. A. Kilby, B. M. Moungey, H. A. S. Underkofler, J. D. Foell, R. C. Balijepalli, T. J. Kamp, C. T. January; Medicine and Physiology, University of Wisconsin-Madison, Madison, WI

Voltage-gated $K^+$ (Kv) channels are essential components of sarcolemmal excitability, and mutations in Kv genes that disrupt the transport of Kv channels in the secretory pathway are associated with several diseases. The molecular mechanisms that govern the intracellular transport (trafficking) of Kv channels in the secretory pathway are unknown. The Sar1-GTPase (Sar1) regulates the transport of proteins out of the Endoplamic Reticulum in coat protein II (COPII) vesicles. Sar1 was cloned using human heart cDNA and was used to generate the dominant negative Sar1[H79G] mutation. We determined the effect of co-expressing Sar1 or Sar1[H79G] and the *human Ether-a-go-go-Related Gene*-encoded $K^+$ channel (Kv11.1) in Human Embryonic Kidney (HEK) 293 cells. Western blot analyses of cells expressing Kv11.1 alone or co-expressing Sar1 demonstrated two distinct Kv11.1 protein bands, a 135kDa protein band that corresponds to the MW of core-glycosylated Kv11.1 and a 155kDa protein band that corresponds to complex-glycosylated Kv11.1. Western blot analyses of cells co-expressing Kv11.1 and Sar1[H79G] demonstrated only the 135kDa core-glycoyslated protein band. We modified the carboxy-terminal of Sar1 to contain a poly-histidine tag (Sar1-poly-his). In cells co-expressing Kv11.1 and Sar1-poly-his, anti-Kv11.1 and anti-poly-his antibodies co-immunoprecipitated Sar1-poly-his and core-glycosylated Kv11.1 respectively. To determine the effect of co-expressing Sar1 or Sar1[H79G] on the sarcolemmal expression of Kv11.1, the peak Kv11.1 current ($I_{Kv11.1}$) was measured in cells expressing Kv11.1 alone, Kv11.1 and Sar1, or Kv11.1 and Sar1[H79G]. Compared to cells expressing Kv11.1 alone, co-expression of Sar1 did not alter the mean peak $I_{Kv11.1}$ (126±29pA/pF and 161±49pA/pF respectively, n=4 each, $p>0.05$). Compared to cells expressing Sar1, co-expression of Sar1[H79G] reduced the mean peak $I_{Kv11.1}$ by 95% (8±4pA/pF, n=4, $p<0.05$). We conclude that Sar1 mediates the trafficking Kv11.1 in the early stages of the secretory pathway.

1235

### Surface Expression of Membrane Proteins Directed by a Novel Group of 14-3-3 Binding Motifs

B. Coblitz,[1] S. Shikano,[1] M. Wu,[1] S. B. Gabelli,[2] L. M. Cockrell,[3] Y. Hanyu,[4] H. Fu,[3] L. M. Amzel,[2] M. Li[1]; [1]Dept. of Neuroscience and HiT Center, Johns Hopkins University, Baltimore, MD, [2]Dept. of Biophysical Chemistry, Johns Hopkins University, Baltimore, MD, [3]Dept. of Pharmacology, Emory University School of Medicine, Atlanta, GA, [4]Institute for Biological Resources and Functions, National Institutes of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan

Dimeric 14-3-3 proteins usually recognize phosphate-specific peptide motifs. With more than two hundred reported interacting proteins, 14-3-3 has been implicated in a range of functions including regulation of subcellular localization. Using a genetic selection, we have identified a group of C-terminal 14-3-3 binding motifs, termed SWTY, which are capable of overriding the strong ER localization signal RXR and redirecting expression on the cell surface. Because the canonical consensus binding motifs of modes I and II are internal, a C-terminal binding consensus, mode III, represents an expansion of the repertoire of 14-3-3-targeted sequences. We report a series of biochemical and mutagenesis studies to investigate the mode III interaction and its function in plasma membrane localization. Because mode III sequences have been found in native proteins, these results support a broad significance of 14-3-3 interaction with protein C-termini.

1236

**Regulation of the RabGAP AS160 by Interaction with 14-3-3 and GLUT4 Vesicle Cargo**

G. Ramm, M. Larance, J. Stockli, D. James; Diabetes and Obesity, Garvan Institute of Medical Research, Sydney, Australia

Insulin stimulates the translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane. Akt-dependent phosphorylation of AS160, a member of the RabGAP/TBC family, has been implicated in this process. Purification of intracellular GLUT4 vesicles and subsequent LC MS analysis revealed the association of AS160 with GLUT4 vesicles. Based on morphological and biochemical criteria, IRAP and Vamp2 are the only other membrane protein constituents in those vesicles. We find that AS160 directly associates with the cytosolic tail of IRAP with high affinity and this facilitates its interaction with GLUT4 vesicles, from which it is released into the cytosol upon phosphorylation. Insulin stimulation of AS160 phosphorylation resulted in binding of AS160 to 14-3-3 proteins. Co-expression of constitutively active Akt and AS160 in CHO cells caused insulin-independent association of AS160 and 14-3-3, implicating a role for Akt in this process. The interaction site was mapped to Thr 642, a known Akt phosphorylation site in AS160. In contrast to AS160WT, an AS160T642A mutant no longer bound 14-3-3 in the presence of insulin and overexpression of this mutant in adipocytes is known to inhibit insulin-stimulated GLUT4 translocation, but to a lesser extent than a mutant lacking three additional Akt phosphorylation sites AS1604P. Preliminary studies indicate that restoration of 14-3-3 binding to this AS160 mutant independently of phosphorylation reversed the inhibitory effect on GLUT4 translocation. Fractionation studies showed that the AS160T642A mutant, in contrast to the AS1604P mutant, underwent insulin-dependent translocation into the cytosol similar to wtAS160. These data suggest a mechanism whereby multiple phosphorylation sites regulate different aspects of AS160 function likely involving either its association with GLUT4 vesicles and/or its GAP activity. This could denote a novel mechanism for protein sorting in eukaryotes regulating cargo RabGAP interaction.

1237

**Akt-phosphorylation Is a Switch for Swty Signals to Direct Cell Surface Expression of Membrane Proteins**

J. L. Chung, S. Shikano, M. Li; Neuroscience and High Throughput Biology Center, Johns Hopkins School of Medicine, Baltimore, MD

Regulated trafficking of receptor proteins is important in controlling many biological processes. SWTY motif represents a novel class of C-terminal transport signals that are capable of overriding ER localization activity and directing protein expression to the cell surface. Phosphorylation-dependent recruitment of 14-3-3 proteins by the SWTY motif is required for SWTY-mediated potentiation of cell surface expression. However, the identity of kinase(s) that phosphorylate the SWTY sequence is unknown. In the present study, we characterize a role for Akt-mediated phosphorylation of SWTY to confer the interaction with 14-3-3. We also report a series of biochemical and cell biology studies to investigate Akt signaling pathway in regulating surface expression via the SWTY motif. Our data suggests that Akt serves as a phosphorylation switch for the SWTY signal to direct membrane proteins to cell surface.

1238

**Targeting Signals in Myelin P0 Protein are Interpreted Differently in MDCK Cells and Neurons**

P. Huang,[1] V. K. Yadav,[2] S. Low,[3] T. Weimbs,[3] B. D. Trapp,[1] G. J. Kidd[1]; [1]Neurosciences, Lerner Institute, Cleveland, OH, [2]Agricultural Research Station, Durgapura, India, [3]Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA

In myelinating Schwann cells, P0 protein is specifically targeted to compact myelin membrane domains and is not detected in other Schwann cell membranes. To determine the molecular basis for P0 targeting we expressed truncated and mutated forms of P0 in MDCK cells. These experiments identified a region of the cytoplasmic C-terminal that was necessary and sufficient for targeting to the basolateral surface. Alanine substitutions identified the main targeting signal as a dual tyrosine-leucine based motif (YAML), in which the tyrosine was essential and the ML motif functional but not necessary. Experiments substituting EGFP for the P0 extracellular domain indicated that secondary apical targeting information was also present in the P0 extracellular domain. To determine whether the tyrosine-based motif predominated in P0 targeting in other polarized cells, we expressed P0 and EGFP-P0 mutated proteins in hippocampal neurons in culture. While many basolaterally targeted proteins in MDCK cells are restricted to the somatodendritic compartment of neurons, P0 was consistently detected in both dendrites and axonal membranes by confocal microscopy. Mutations to the tyrosine motif did not alter P0 distribution. EGFP-P0 chimeras were restricted to the neuronal ER. These data indicate that P0 contains a hierarchy of targeting signals that are utilized differently in different polarized cells.

1239

**The Cysteine-Rich Domain of the Secreted Proprotein Convertases PC5A and PACE4 Functions as a Cell Surface Anchor through Interaction with Tissue Inhibitors of Metalloproteinases**

G. Mayer,[1] N. Nour,[1] J. S. Mort,[2] C. M. Overall,[3] N. G. Seidah[1]; [1]Biochemical Neuroendocrinology, Montreal Clinical Research Institute, Montreal, PQ, Canada, [2]Joint Diseases Laboratory, Shriners Hospital for Children, Montreal, PQ, Canada, [3]Oral Biological and Medical Sciences, University of British Columbia, Vancouver, BC, Canada

The proprotein convertases PC5, PACE4 and furin contain a C-terminal cysteine-rich domain (CRD) of unknown function. We demonstrate that the CRD confers to PC5A and PACE4 properties of protein-protein interactions and cell surface tethering. Confocal microscopy and biochemical analysis revealed that the CRD is essential for cell surface localization of PC5A and PACE4 and that it co-localizes and co-immunoprecipitates with the full length and C-terminal domain of the tissue inhibitor of metalloproteinases-2 (TIMP-2). Moreover, in TIMP-2 null fibroblasts PC5A is not retained at the cell surface unless TIMP-2 is re-introduced by co-expression, indicating that the formation of a complex between both proteins

is requisite for plasma membrane anchorage of the convertase. Further analysis revealed that both PC5A and PACE4 can bind different sets of TIMP family members and that PC5A binds TIMP-3 in the extracellular matrix. In addition, to extend the relevance of proprotein convertases and TIMPs interaction towards the realm of a whole animal, PC5A and TIMP-2 are shown to be expressed in the small intestine and liver and to co-localize over the surface of enterocytes of the villi and crypts found in the duodenum and jejunum, as well as in liver sinusoids. In conclusion, the CRD of PC5A and PACE4 may represent a nucleation point favoring formation of complexes with TIMPs which could have a direct impact on the cell surface interaction of these convertases with their cognate substrates including lipoprotein and endothelial lipases and possibly matrix metalloproteases.

1240

**Interferon-gamma Promotes Membrane Retention of NHE1 and Associated Proteins Ezrin, Radixin and Moesin via STAT1 in HT-29 Cells**

F. Magro, S. Fraga, P. Soares-da-Silva; Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal

This study evaluated the effect of interferon-γ (IFN-γ) upon the function and expression of type 1 $Na^+/H^+$ exchanger (NHE1) in human intestinal epithelial HT-29 cells, namely that concerning the abundance of surface NHE1 and NHE1 binding to the ezrin, radixin, and moesin (ERM) family of proteins. HT-29 cells express endogenous NHE1 and the ERM family of proteins that retain the localization of NHE1 in the membrane. Long-term exposure (24 h) of HT-29 cells to IFN-γ resulted in a concentration dependent decrease in NHE1 activity. Inhibition of NHE1 activity by IFN-γ was absent after pretreatment with cariporide. The long-term exposure to IFN-γ was accompanied by increase in surface NHE1 and ERM abundance and no changes in total NHE1 and ERM abundance. Inhibition of STAT1 with epigallocatechin-3-gallate (EGCG) prevented the inhibitory effect of IFN-γ. Treatment with IFN-γ activated phospho-STAT1 was markedly attenuated by EGCG. The IFN-γ-induced increase in surface NHE1 and ERM abundance was prevented by EGCG. In conclusion, long-term inhibition of NHE1 activity by IFN-γ involves STAT1 phosphorylation and is accompanied by increased abundance of surface NHE1 and the NHE1 membrane anchoring ERM proteins.

1241

**Calcineurin Homologous Protein (CHP) Regulates $Na^+/H^+$ Exchanger-3 (NHE3) Apical Delivery in Epithelial Cells**

F. Di Sole,[1,2] V. Babich,[3,2] O. W. Moe[2]; [1]Cell and Molecular Biosciences, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom, [2]Internal Medicine, Nephrology Division, University of Texas Southwestern Medical Center, Dallas, TX, [3]Molecular Neuroendocrinology, National Institute for Medical Research, London, United Kingdom

Asymmetric membrane transporters delivery is an essential function in polarised cells. CHP is a widely expressed and evolutionary conserved $Ca^{2+}$-binding protein required for constitutive membrane traffic. CHP binds the apical transmembrane protein NHE3, a key mediator of net sodium and fluid balance in intestinal and renal epithelia. We have recently shown that this binding regulates NHE3 function in response to adenosine; here, we report a key role of CHP in NHE3 apical translocation. In opossum kidney (OK) cells, NHE3 is located in apical clusters where it associates with F-actin filaments. CHP co-localizes with NHE3 in the clusters (immunofluorescence). Overexpression of CHP increases both NHE3 activity (microfluorimetry) and apical surface NHE3 antigen (biotinylation assay) by 50%. Knockdown of CHP by siRNA inhibits NHE3 activity and reduces apical surface NHE3 antigen by 50%. CHP knockdown also decreases NHE3 cluster formation and F-actin polymerization. In order to examine the contribution of CHP-NHE3 binding to the process, GFP tagged NHE3 mutant lacking CHP binding region ($NHE3^{\Delta 462\text{-}552}$) was expressed in OK cells. $NHE3^{\Delta 462\text{-}552}$ does not show the typical apical membrane targeting seen in wild type NHE3 but localized uniformly in the cytoplasm, suggesting that lack of the CHP-NHE3 interaction induces mistargeting of NHE3 to the apical membrane. Consistent with a central role for ezrin-dependent NHE3 translocation, overexpression of CHP clearly induces a strong increase in phospho-ezrin (immunofluorescence and SDS-PAGE) without changes in total ezrin. CHP knockdown dramatically reduces phospho-ezrin (immunofluorescence and SDS-PAGE). In conclusion, CHP plays a critical role in NHE3 delivery to the apical membrane and its clustering organization. Ezrin phosphorylation may mediate the CHP-induced NHE3 apical translocation.

1242

**Curcumin Increases the CFTR Expression by Down-Regulating Calreticulin, a Negative Regulator of CFTR**

K. Harada,[1] T. Okiyoneda,[1] Y. Hashimoto,[1] K. Ueno,[1] K. Nakamura,[2] K. Yamahira,[1] T. Sugahara,[1] T. Shuto,[1] I. Wada,[3] M. S. Suico,[1] H. Kai[1]; [1]Molecular Medicine, Faculty of Pharmaceutical Sciences, Kumamoto, Japan, [2]Pediatrics, Faculty of Medical and Pharmaceutical Sciences, Kumamoto, Japan, [3]Cell Science, Fukushima Medical University School of Medicine, Fukushima, Japan

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, is caused by the mutation of CFTR, which functions as a cAMP-dependent $Cl^-$ channel at the plasma membrane of epithelial cells. The most common mutant of CFTR in CF patients, ΔF508 CFTR, has a trafficking defect from the ER although it can function as a $Cl^-$ channel, if expressed at the plasma membrane. Thus, rescue of ΔF508 CFTR from ER retention is a potential therapeutic strategy for CF. Recently, it was reported that curcumin corrects the defective trafficking of CFTR mutant (ΔF508). However, the effect of curcumin is under debate and its mechanism of action remains unknown. Here, we show that curcumin down-regulates an ER chaperone, calreticulin (CRT), which is a novel negative regulator of CFTR trafficking, leading to the increase of CFTR expression. Moreover, curcumin and CRT knockdown significantly enhanced the function of ΔF508 CFTR rescued by 26°C incubation. These results indicate that curcumin stimulates the trafficking of rescued ΔF508 CFTR to the plasma membrane by decreasing CRT, a negative regulator of CFTR. These findings have important implications in developing therapeutic approaches that target the trafficking of CFTR.

1243

**Hypotonicity Induces Actin Remodeling Associated with Aquaporin-2 Internalization and Cytosol to Membrane Translocation of ICln in Collecting Duct Renal Cells**

G. Tamma,[1] G. Procino,[1] A. Strafino,[1] M. Paulmichl,[2] M. Svelto,[1] G. Valenti[1]; [1]Department of General and Environmental Physiology, University of Bari, Bari, Italy, [2]Department of Biomolecular Science and Biotechnology, University of Milan, Milan, Italy

Kidney tubule cells are subjected to a significant transepithelial osmotic gradient and have a strong requirement for cell volume regulatory mechanisms. ICln is essential for the generation of ion currents activated during regulatory volume decrease after cell swelling (RVDC). Hypotonic

treatment (100 mOsm, 10 min) lead to a significant transposition of ICln to the cell membrane while increased the amount of AQP2 localized intracellularly. Interestingly, the total amount of AQP2 phosphorylated at ser-256, decreased significantly after hypotonic shock. Since the cAMP dependent protein kinase A (PKA), is committed to phosphorylate AQP2, we next examined the spatio-temporal intracellular dynamics of cAMP using fluorescence resonance energy transfer (FRET) technique in living cells. Interestingly we found that hypotonic stress caused decreased concentration of cAMP. Moreover, FRET analysis and co-immunoprecipitation experiments revealed that hypotonicity increased the interaction of ICln with actin, which might be important for its insertion into the membrane. Hypotonicity caused a deep actin reorganization, consisting in the loss of stress fibers and the formation of F-actin patches at the cell border. The expression of the dominant negative of Cdc42 (N17-Cdc42) suppressed the hypotonicity-induced microspikes formation at the cell border suggesting that Cdc42 principally regulates actin rearrangement. Hypotonic treatment activated LIMK1, a known downstream effectors of Cdc42, and increased the phosphorylation of the ADP-depolymerization factor cofilin, a known downstream effector of LIMK1. Phosphorylation inactivates cofilin, resulting in formation of F-actin patches at the cell border. Together these data demonstrate that hypotonicity causes a deep actin remodeling, increases the total amount of AQP2 localized intracellularly, and activates translocation of ICln to the plasma membrane. All these effects may contribute to regulatory volume decrease after hypotonicity induced cell swelling in renal medulla.

1244

**Kv21 K+ Channels Form Dynamic Cell Surface Microdomains That Are Sites For Membrane Insertion**

K. M. S. O'Connell, A. S. Rolig, M. M. Tamkun; Biomedical Sciences, Colorado State University, Fort Collins, CO

Voltage-gated $K^+$ (Kv) channels play a key role in establishing the resting membrane potential, shaping action potential repolarization and regulating spike frequency in nerve and muscle. These channels are often targeted to specific cell regions where they are assembled into signaling complexes. However, little is known about how these channels are transported to the membrane or about their behavior once they arrive. Using fluorescent protein tagged channels, FRAP and time lapse confocal microscopy, we show that the delayed rectifier Kv2.1 is preferentially inserted in specific regions of the HEK cell plasma membrane. These regions, which range in size from 0.1 $\mu m^2$ to > 7 $\mu m^2$ (avg = 0.86 ± 0.1 $\mu m^2$), are dynamic, often fusing and breaking apart over several minutes. Channels were delivered to these surface clusters via small intracellular transport vesicles that were observed fusing with preexisting cluster domains. Once inserted into a cluster, channel moved freely within that domain. The inability of channel to cross the cluster boundary is reminiscent of the “picket fence” model of membrane diffusion barriers. Kv2.1 containing surface clusters were similar in appearance to cholera toxin (Ctx) labeled GM1-ganglioside enriched lipid rafts, however the Kv2.1 clusters rarely internalized over the time course of Ctx internalization, nor was there any colocalization between the two domains. Cholesterol depletion with 2-hydroxypropyl-cyclodextrin decrease Kv2.1 mobility within the cluster ($\tau_{con}$ = 14.1 s vs. $\tau_{CD}$ = 53.1 s) and slowed cluster dynamics within the entire plasma membrane. However, Kv2.1 cluster domains were resistant to detergent solubilization under conditions expected to disrupt raft lipid (0.2% TX-100 + 7.5 mM octylglucoside at 30°C). Therefore, Kv2.1-containing surface clusters appear to be specialized microdomains for membrane insertion whose maintenance is likely a combination of protein-lipid and protein-protein interactions.

1245

**Plasma Membrane Association of K-ras4B is Dynamic**

J. R. Silvius, P. Bhagatji, R. Leventis, D. Terrone; Biochemistry, McGill University, Montréal, PQ, Canada

We have adapted the phenomenon of rapamycin-induced protein heterodimerization, originally exploited by Schreiber and colleagues as a ‘chemical switch,’ to investigate the kinetics of association of lipid-modified proteins with their target membranes in living cells. Lipidated fluorescent protein constructs that incorporate a rapamycin-dependent heterodimerization domain, and that are reversibly anchored to various cellular membranes, translocate from these membranes to mitochondria upon addition of rapamycin to cells coexpressing a mitochondrial outer membrane protein that incorporates a complementary heterodimerization domain. By quantitative image analysis the kinetics of the translocation process, and thereby the rates of dissociation of the fluorescent proteins from their normal target membranes, can be determined. Using this approach we show that singly-lipidated proteins lacking additional membrane targeting determinants can very rapidly redistribute between different cellular membranes, while analogous constructs bearing multiple lipid groups (S-prenyl or N-myristoyl plus S-acyl residues), show much slower rates of redistribution, with kinetics that appear to reflect the rates of turnover of the protein-linked S-acyl residues. Interestingly, we find that fluorescent fusion constructs incorporating full-length K-ras4B, constitutively activated or activation-resistant mutants of this protein or the prenylated/polybasic K-ras4B plasma membrane-targeting sequence alone are reversibly associated with the plasma membrane and can redistribute from the plasma membrane to mitochondria with halftimes of the order of a few minutes. The dynamic nature of the plasma membrane targeting of K-ras4B could in principle contribute additional versatility to the function of this protein, as does the acylation/deacylation-dependent redistribution of H- and N-ras between different cellular compartments. The novel method described here may offer a valuable alternative to fluorescence-photobleaching/photoactivation methods to investigate the dynamics of association of proteins with cellular membranes, particularly for proteins that are reversibly but very strongly associated with their target membranes. (Supported by the Canadian Institutes of Health Research).

1246

**Subcellular Localization and Signaling Roles of Human Ras Superfamily Small Gtpases**

W. D. Heo, T. Meyer; Molecular Pharmacology, Stanford, Palo Alto, CA

The proteome of mammalian cells is largely built from protein families with individual members carrying out specific cellular tasks. Here we focus on the question of how the Ras superfamily regulate different signaling processes by cloning 142 human small GTPases and generating constitutively active and dominant forms for each of them. We first analyzed the subcellular localization of the wild-type and constitutively active forms of each small GTPases in HeLa and NIH3T3 and identified 48 small GTPases that are at least partially localized to plasma membrane (PM). Based on their PM localization and localization motifs, we classified them into four groups and characterized the localization mechanisms for each of the groups. Three of the groups included polybasic regions and we focused therefore on the role of these C- and N-terminal polybasic motifs as the most common mediators of the PM localization. Our initial studies suggest that electrostatic interaction with the PM, presumably by binding to phosphatidylserine, and not binding to $PI(4,5)P_2$ lipids is responsible for the PM targeting. In addition to the localization study, we also investigated the activation of PI3kinase by co-expressing a $PIP_3$ biosensor (Akt-PH domain) with each of the constitutively active human small GTPase. We

identified over 30 small GTPases that activate the PI3K pathway and explored whether some of them may participate in a positive feedback loop as has been suggested for Rac and CDC42. Using experiments in which we used dominant negative small GTPases and the PI3K inhibitor LY294002, we now identified a number of additional small GTPases that may participate up-stream and within such a positive feedback loop. Together, both these projects provide us with a better understanding of how functional specificity has evolved within a large family of signaling proteins.

## Protein Folding & Assembly in the Endoplasmic Reticulum I (1247-1259)

1247

### The Recognition of a Dibasic Degradation Motif at the Endoplasmic Reticulum

A. Ray, S. High; Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

Misfolded proteins that fail to pass the Endoplasmic Reticulum (ER) Quality Control system are ultimately delivered to cytosolic proteasomes for degradation by the ER-associated degradation (ERAD) pathway. Key events during ERAD include: substrate selection, retro-translocation and proteolysis of the misfolded proteins. The degron is a dibasic amino acid motif located within the hydrophobic transmembrane domain of some membrane proteins, such as TCR-α, and has been shown to target so called 'orphan' subunits and mis-assembled complexes for cellular degradation. The degron is masked when the subunits are correctly assembled into authentic oligomeric complexes at the ER. The ability of the degron to promote degradation is well documented, but the molecular basis for its recognition, and the route by which this substrate is delivered to the degradation pathway are unknown. The aim of this study is to investigate in detail the molecular mechanism underlying degron function. In this study, we have used both *in vivo* and *in vitro* techniques to investigate the effects of the degron when it is introduced into a model polytopic membrane protein, bovine opsin. Electron microscopy and immunofluorescence data indicates that wild type opsin is correctly sorted to the cell surface, whilst a degron mutant is retained within the ER, consistent with its targeting for degradation. Pulse Chase analysis indicates that the opsin degron mutant is degraded and that this process is mediated by proteasomes. Therefore the insertion of a degron into a polytopic membrane protein leads it to its specific ER retention and degradation via a route that closely resembles that used by the TCR-α subunit. The use of cross-linking has begun to reveal differences in the membrane environment resulting from the introduction of the degron.

1248

### Retro-translocation of a Fluorescent-labeled ERAD Substrate through the ER Membrane *in vitro* Requires ATP and both Lumenal and Cytosolic Proteins

J. Wahlman,[1] W. R. Skach,[2] J. L. Brodsky,[3] A. E. Johnson[4]; [1]Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, [2]Division of Molecular Medicine,, Oregon Health Sciences University, Portland, OR, [3]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, [4]Department of Biochemistry and Biophysics, Chemistry, Medical Biochemistry & Genetics, Texas A&M University, College Station, TX

Endoplasmic reticulum-associated degradation (ERAD) involves the recognition of misfolded proteins in the endoplasmic reticulum (ER) lumen and their transport back to the cytosol for degradation. A number of proteins have been proposed to participate in this process, but the exact role of each has been difficult to establish. We have therefore created an *in vitro* system to assess the contributions of individual components to retro-translocation. After canine pancreatic microsomes were treated with high pH to release their lumenal contents, the extracted microsomes were reconstituted with a fluorescent-labeled ERAD substrate, the non-glycosylated yeast mating pheromone (Δgpαf), and either total lumenal proteins or some combination of individual purified lumenal proteins (e.g. BiP, PDI, etc.). The reconstituted microsomes containing encapsulated BODIPY-Δgpαf were then incubated in the presence of BODIPY-specific antibodies, and in the presence or absence of reticulocyte lysate, nucleotides, or various purified cytosolic components. Since the BODIPY fluorescence intensity is quenched about 80% by its binding to the antibody, the release of the encapsulated BODIPY-Δgpαf from the interior of the microsomes can be followed as a function of time by monitoring the reduction of BODIPY emission. In the presence of reticulocyte lysate, ATP, and total lumenal proteins more than 60% of the BODIPY-Δgpαf is retro-translocated within 30 minutes at 30 $^{\circ}$C under our *in vitro* conditions. In the absence of any of these components, only background exposure of encapsulated BODIPY-Δgpαf to the cytosolic antibody is observed. This novel spectroscopic approach therefore reveals the real-time rate of retro-translocation and its dependence upon molecules on both sides of the mammalian ER membrane. The importance of various lumenal and cytosolic components is now being determined. (Supported by NIH grant GM26494 and the Welch Foundation).

1249

### Monitoring the ER Luminal and the Cytosolic Face of the ERAD Substrate CD3delta

H. Lorenz, J. Lippincott-Schwartz; CBMB, NIH, NICHD, Bethesda, MD

Proteins of the secretory pathway, which fail to pass quality control are retrotranslocated from the endoplasmic reticulum into the cytosol and eventually degraded by the proteasome. This process is called ER-associated degradation (ERAD). Here, we examined the fate of both the luminal and cytosolic domains of the well-described ERAD substrate CD3delta using a new live cell imaging strategy. Different fluorescent proteins were placed simultaneously at the N- and C-terminus of CD3delta. By doing so, the protein's fate on both sides of the ER membrane can be observed in live cells. Furthermore, because a fluorescent protein's ability to fluoresce depends upon its proper fold, information about the folding state on either side of the membrane can be obtained. Using this chimera, we were able to demonstrate that CD3delta accumulates not only in the cytosol, but also significantly within the ER membrane upon proteasomal inhibition. Interestingly, while accumulated CD3delta remained fully folded along the membrane, its fluorescence was completely abolished in the cytosol, indicating a mere unfolded cytosolic pool of CD3delta. The application of drugs compromising sugar processing severely affected the fate and folding of CD3delta on either side, which could be detected and quantified. The strategy of doubly labeled transmembrane proteins expands significantly the use of fluorescent fusion proteins and helps understanding the steps proteins undergo while crossing membranes.

1250

### ERAD Substrates Traffic from the ER Due to ER Export Signals: ER Retention of Misfolded Proteins Is Due to a Failure to Exit

M. M. Kincaid, A. A. Cooper; School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO

The endoplasmic reticulum (ER) is the site of entry for soluble and transmembrane proteins into the secretory pathway of all eukaryotic cells. Proteins are translocated into the ER where they fold, are modified by carbohydrate addition and disulfide bond formation, and assemble into complexes. Misfolded proteins in the ER are retained and degraded by ER-associated degradation (ERAD). However, a subset of ERAD substrates fails to be retained in the ER and instead traffics to the Golgi prior to degradation. Our study in *Saccharomyces cerevisiae* of model ERAD substrates, with or without defined ER exit signals, demonstrates that misfolded proteins depart the ER by presenting functional exit signals in the same manner as the corresponding correctly folded proteins. Conversely, ERAD substrates that remain in the ER likely lack ER exit signals or are unable to functionally present them. These data indicate that ER retention of misfolded proteins is unlikely an active process but instead reflects a failure to exit the ER. These findings are contrary to the prevailing view of stringent and active ER quality control (ERQC) mechanisms preventing misfolded and potentially toxic proteins from traversing the secretory pathway. In addition, these data highlight the dynamic interplay between ERAD and ER exit in which both processes compete for binding of misfolded proteins that contain ER export signals. Inhibition of ERAD results in greater ER export of such misfolded proteins while blocking ER exit results in enhanced degradation of these proteins by ERAD.

1251

### A Screening System for Compounds to Protect Cells against Endoplasmic Reticulum (ER) Stress

Y. Tabata, O. Hori, K. Takano, Y. Kitao, S. Ogawa; Neuroanatomy, Kanazawa University Graduate School of Medicine, Kanazawa City, Japan

Once cells are exposed to stresses such as glucose starvation, inhibition of protein modification, disturbance of $Ca^{2+}$ homeostasis and oxygen deprivation, unfolded proteins accumulate in the endoplasmic reticulum (ER stress). If protein loads in the ER exceed its folding capacity, or dome defects in the ER stress response exist, cells tend to die, typically, with apoptotic features (ER stress-induced cell death). The crucial roles of the ER stress response/ER stress-induced cell death have been reported in many pathological conditions including diabetes, arteriosclerosis, ischemia and neurodegenerative diseases. Herp is a membrane bound, ubiquitin-like protein that is located in the endoplasmic reticulum (ER). Although Herp is strongly induced by ER stress, it decays rapidly consequent to proteasome-mediated degradation, suggesting the possible contribution of Herp in the ERAD. We recently reported that targeting disruption of the Herp gene caused F9 embryonic carcinoma cells vulnerable to ER stress, and the ER stress-induced death in F9 Herp null cells was associated with the aberrant ER stress signaling, structural changes in the ER and caspase activation (Genes Cells 2004, 9, 457-469). Using these cell lines and MIN6 cells, mouse insulinoma cell lines, we developed a screening system to obtain molecules that suppress ER stress/ER stress-induced cell death. Among approximately 20 well-known compounds, Dantrolene and some anti-oxidants had protecting effects against ER stress. We speculate that this system could provide novel therapeutic targets to the ER stress-related diseases.

1252

### The Putative Protein Determinant for ER-Associated Degradation

K. Kanehara, A. Sayeed, E. D. Spear, D. T. W. Ng; Temasek Life Sciences Laboratory and Department of Biological Sciences, National University of Singapore, Singapore, Singapore

The maturation of new synthesized proteins entering the secretory pathway is monitored by ER quality control (ERQC) mechanisms. ERQC retains newly proteins as they are folding. Proteins that are irreversibly misfolded are sorted and degraded by the ER-associated protein degradation (ERAD) pathway. To understand the relationship between quality control and ERAD, we analyzed a misfolded variant of carboxypeptidase Y (CPY) termed CPYΔ1. CPYΔ1 lacks the C-terminal 154 amino acids of CPY. CPYΔ1 was recognized and retained by ER quality control but failed to enter the ERAD pathway. The observation suggested that the deletion disrupted an ERAD-specific targeting signal. We showed that a single context-specific N-linked glycan is a required determinant of the signal. An analogous signal was found in misfolded proteinase A (PrA*), but in a different position. We hypothesized from these data that substrates utilize a bipartite signal comprised of carbohydrate and peptide elements. To test our hypothesis, systematic deletion analysis was performed on CPY* with the aim of disrupting the peptide determinant. Deletions spanning most of the protein had no effect on degradation. However, a small protein domain was determined to be required for ERAD, even with the signal carbohydrate intact. Interestingly, the determinant is functional when moved to another part of the molecule suggesting that it is not positionally constrained. These data indicate that specifically embedded signals in glycoproteins are used for targeting to the ERAD pathway if misfolded.

1253

### How UPR Regulation of the BiP/Kar2p Chaperone Contributes to ER Stress Tolerance

C. Hsu, D. T. W. Ng; Temasek Life Sciences Laboratory, Singapore, Singapore

The endoplasmic reticulum (ER) provides a tightly regulated environment for the folding and maturation of proteins destined to enter the secretory pathway. The homeostasis of the ER can be perturbed under various stress conditions, leading to the accumulation of misfolded and unfolded protein in the ER (termed ER stress). Consequently, a stress-inducible pathway known as unfolded protein response (UPR) is activated to alleviate stress. The UPR is an ER-to-nucleus signal transduction pathway that regulates a wide variety of genes to restore cellular homeostasis. However, how the induction of individual UPR targets contributes to stress tolerance has not been determined. In this study, we analyzed the role of *KAR2* gene activation. *KAR2*, a major UPR target gene, encodes the *Saccharomyces cerevisiae* ortholog of BiP, an ER chaperone of the Hsp70 family. We constructed a strain by which the *KAR2* gene is expressed at basal levels but unresponsive to the UPR (called *UPRE$^d$-KAR2*). The strain (CHY220) showed growth sensitivity to ER stress caused by the glycosylation inhibitor tunicamycin and overexpression of the misfolded protein CPY*. Interestingly, the strain tolerated a challenge by dithiothreitol (DTT), a reducing agent that disrupts disulfide bonds in the ER. These data show that *KAR2* upregulation is required for some forms of ER stress and not others. Kar2p plays a variety of essential functions including protein folding, translocation, and the turnover of misfolded proteins. Using this system, we are currently determining the specific role that *KAR2* upregulation plays in ER stress tolerance.

1254

### Overexpression of Megsin Induces Neuronal Degeneration: Role of the Endoplasmic Reticulum Stress Response

K. Takano,[1] Y. Kitao,[1] R. Inagi,[2] T. Miyata,[2] O. Hori,[1] S. Ogawa[1]; [1]Department of Neuroanatomy, Kanazawa University Graduate School of Medicine, Kanazawa, Japan, [2]Molecular and Cellular Nephrology, Institute of Medical Sciences, Tokai University, Isehara City, Japan

Serpinopathies are caused by intracellular accumulation/polymerization of mutant serine protease inhibitors. Transgenic rats overexpressing megsin (Tg meg), a newly identified serine protease inhibitor (serpin), unexpectedly demonstrated impaired memory by the water maze test. Hippocampal neurons cultured from Tg meg rats displayed enhanced sensitivity to glutamate. Consistently, immunoblotting of hippocampal extracts from Tg meg rats showed enhanced levels of ER stress proteins along with the activation of caspase-12 and caspse-3, which peaked 4-6 months after birth, which was associated with decreased neuronal density in the CA1 region of Tg meg animals. Immunohistochemical analysis of the hippocampus revealed increased expression of ER stress proteins, as well as activated caspase-3 and caspase-12. Similar findings of ER stress, activation of caspases and decreased neuronal density were also observed in tyrosine hydroxylase-positive nigro-striatal neurons, accompanied with the impairement of motor coordination. These data suggest that overexpression of megsin results in ER stress, which eventuates in selective cellular dysfunction and death, providing a novel model; increased levels of a wild-type serpin induce dysfunction of vulnerable neuronal populations.

1255

**Regulation of Endoplasmic Reticulum (ER) Stress at Translocational Site into the ER**

O. Hori,[1] M. Miyazaki,[1] E. Hartmann,[2] P. Mai,[2] S. Ogawa[1]; [1]Department of Neuroanatomy, Kanazawa University, Graduate School of Medicine, Kanazawa City, Japan, [2]Universität Lübeck, Lübeck, Germany

Stress-associated ER protein 1 (SERP1), also known as Ribosome-associated membrane protein 4 (RAMP4), is a Sec61-assocaited polypeptide that is induced by endoplasmic reticulum (ER) stress. $SERP1^{-/-}$ mice, made by targeted gene disruption, demonstrated growth retardation, increased mortality and impaired glucose tolerance. Consistent with high levels of SERP1 expression in pancreas, pancreatic islets from $SERP1^{-/-}$ mice failed to rapidly synthesize proinsulin in response to a glucose load. Unexpected was strong expression of SERP1 in the anterior pituitary. Reduced size and enhanced ER stress were observed in the anterior pituitary of $SERP1^{-/-}$ mice, and growth hormone production was slowed in $SERP1^{-/-}$ pituitary after insulin stimulation. Experiments using pancreatic microsomes from $SERP1^{+/+}$ and $SERP1^{-/-}$ mice revealed aberrant association of ribosome and Sec61 complex, and enhanced ER stress in $SERP1^{-/-}$ pancreas. In basal condition, Sec61 complex in $SERP1^{-/-}$ microsomes were more co-fractionated with ribosomes, compared with $SERP1^{+/+}$ counterparts, in high-salt condition. After glucose stimulation, in contrast, they showed less co-fractionation at early periods (45min), but more at late periods (120min). Although intracellular insulin/proinsulin levels were not significantly changed in both genotypes, these results suggest that subtle changes in translocation efficiency play an important role in the control of ER stress and rapid polypeptide synthesis.

1256

**Analysis of UPR Genes in Antibody Secretion: Chop Downregulation Enhances Antibody Specific Productivity**

G. Bannish, M. Rycyzyn, W. Cornwell, T. Petley, K. Stark, B. Pikounis, J. Giles-Komar; Cellular Biology, Centocor, Radnor, PA

The homeostasis of the ER is regulated in part by the unfolded protein response (UPR). By analyzing a panel of antibody secreting myelomas, a correlation was made between higher specific productivities and increased steady state levels of RNA for the UPR genes Bip, CHOP, and activated Xbp-1 (X54). This correlation was sustained when cells underwent stress, either by pharmacologic induction or the end static phase of their growth curve. Since increased levels of CHOP have previously been reported to inhibit protein production and induce apoptosis, we sought to ascertain if decreasing CHOP levels in cells could increase specific productivities. To that end, a representative cell line, cell line A, was stably transfected with shRNA vectors to target CHOP, Bip, or vector controls. Of the stable cell lines generated, 92% of the ones with the highest levels of secreted antibody were derived from the CHOP shRNA transfectants. Upon further analysis, these clones were found to express reduced amounts of CHOP RNA as assessed by Taqman analysis and had specific productivity values 2.5 fold higher than vector controls. This finding is consistent with the alternative UPR induction found to occur in plasma B cells and suggests a non-apoptotic mechanism for CHOP-mediated reduction of protein secretion.

1257

**Differential Accumulation of Derlin RNAs and Proteins during Prolonged ER Stress**

M. E. Kirst,[1] R. Jung,[2] R. S. Boston[1]; [1]Botany, NCSU, Raleigh, NC, [2]Pioneer Hi-Bred, A DuPont Company, Johnston, IA

Maintenance of a functional protein secretory pathway is facilitated by removal of terminally misfolded proteins through an ER-associated degradation pathway (ERAD). We have identified four maize genes (ZmDerlin1-1, 1-2, 2-1, 2-2) that encode proteins conserved in sequence and core function with the yeast ERAD protein, Der1p. Both ZmDerlin1 and ZmDerlin2 proteins are present in ER of the endosperm and are also associated with protein bodies, the ER derived protein storage organelles of the seed. Quantitative RT-PCR analysis showed that ZmDerlin1-1, 1-2 and 2-1 were up-regulated by ER stress while ZmDerlin2-2 expression appeared to be unaffected. During prolonged ER stress, we observed a reduction in the accumulation of ZmDerlin1 protein, but not ZmDerlin2 or the molecular chaperone BiP. RT-PCR analysis indicated that this reduction was not reflected in the polysomal RNA population. Thus, ZmDerlin1 itself may be an ERAD substrate.

1258

**Human Homologs of Ubc6p Ubiquitin-conjugating Enzyme and Phosphorylation of Hsubc6e in Response to ER Stress**

R. Oh,[1,2] X. Bai,[1] J. Rommens[1,2]; [1]The Hospital for Sick Children, Toronto, ON, Canada, [2]Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada

Ubiquitin-conjugating enzyme Ubc6p is a tail-anchored protein that is localized to the cytoplasmic face of the ER membrane and has been implicated in the degradation of many misfolded membrane proteins in yeast. We have undertaken characterization studies of two human homologs, hsUbc6 and hsUbc6e. Both possess a tail-anchored protein motif, display high conservation in their catalytic domain, and are functional ubiquitin-conjugating enzymes as determined by *in vitro* thiol-ester assay. Both also display induction by the unfolded protein response, a feature of many endoplasmic reticulum associated degradation (ERAD) components. Post-translational modification of hsUbc6e was identified as phosphorylation and observed to be ER stress related. Under stress by thapsigargin and dithiothreitol, phosphorylation was diminished in mouse embryonic fibroblasts deficient for the ER stress regulated kinase PERK, suggesting that Ubc6e is regulated by this kinase. The phosphorylation site was mapped to Ser184, which resides within the uncharacterized region linking the highly conserved catalytic core and the carboxyl terminal transmembrane domain. Phosphorylation of hsUbc6e was dynamic and did not alter stability or subcellular localization. Parkin, a known Ubc6e

interacting E3 ligase, was found by co-immunoprecipitation experiments to associate with either phosphorylated or unphosphorylated forms of hsUbc6e. Assays of hsUbc6e$^{S184D}$ and hsUbc6e$^{S184E}$, which mimic the phosphorylated state, suggest that phosphorylation may reduce capacity for forming ubiquitin-enzyme thiol-esters. The occurrence of two distinct Ubc6p homologs in vertebrates, including one with phosphorylation modification in response to ER stress, emphasizes diversity in function between these E2s during ERAD processes.

1259

### Calnexin Is Important for Inhibition of the ERAD of Incompletely Folded CFTR but Not for the ER Retention

T. Okiyoneda,[1] A. Niibori,[1] K. Harada,[1] I. Wada,[2] M. Ikawa,[3] T. Kouno,[1] T. Shuto,[1] M. S. Suico,[1] H. Kai[1]; [1]Molecular Medicine, Kumamoto Univ, Kumamoto, Japan, [2]Cell Science, Fukushima Medical Univ, Fukushima, Japan, [3]Genome Information Research Center, Osaka Univ, Osaka, Japan

DeltaF508 CFTR, which is the most common mutant of CFTR in Cystic fibrosis (CF) patients, is retained in the ER by the ER quality control system, leading to ER-associated degradation (ERAD). Since DeltaF508 CFTR is functional as a regulated $Cl^-$ channel when expressed at the plasma membrane, rescue of DeltaF508 CFTR from ER retention may be useful for CF therapy. However, the molecular mechanism of the ER retention remains unclear although an ER chaperone, calnexin is thought to participate in the ER retention of DeltaF508 CFTR. To clarify whether calnexin retains DeltaF508 CFTR in the ER, we analyzed the effect of calnexin mutant, which is exported from the ER. Our results indicate that DeltaF508 CFTR is retained in the ER even if calnexin is exported from the ER. In calnexin-deficient cells, DeltaF508 CFTR was still retained in the ER and quickly degraded by the ERAD pathway. However, the introduction of calnexin into these cells inhibited the ERAD of DeltaF508 CFTR. These results demonstrate that calnexin is required for inhibition of the ERAD of DeltaF508 CFTR but not for the ER retention.

## Mechanisms of Nuclear Transcription (1260-1278)

1260

### NF-*k*B/RelA Transcription Factor Interacts with *a*-Actinin Isoforms

V. Babakov,[1] O. Petukhova,[1] L. Turoverova,[1] I. Kropacheva,[1] D. Tentler,[1] E. Podolskaya,[2] K. I. Magnusson,[3] G. Pinaev[1]; [1]Cell Cultures, Institute of Cytology, St. Petersburg, Russian Federation, [2]Environmental Biomedical Mass Spectrometry, Analytical Instrumentation, St. Petersburg, Russian Federation, [3]Medical Microbiology, Linkoping University, Linkoping, Sweden

The NF-kB/RelA family of transcription factors regulates inducible transcription of a large number of genes in response to diverse stimuli. Little is known, however, about location of NF-κB in cytoplasm and transport mechanism to the nucleus. We have found that NF-κB is associated with actin-binding protein α-actinin-4 by several criteria. First, NF-κB and α-actinin-4 co-localized along actin stress fibers and in focal adhesions in A431 cells. EGF- and TNF-α-stimulation of A431 cells for 5 min lead to a dramatic actin cytoskeleton reorganization, dorsal ruffling formation and accumulation of F-actin, α-actinin and p65/RelA subunit of NF-κB in this area. After 30-min stimulation, α-actinin-4 and p65 were found in the nucleus. Pretreatment of the cells with cytochalasin D and wortmannin prior to EGF or TNF-α treatment lead to increased p65 and α-actinin-4 translocation. Co-localization of α-actinin-4 with p65 was confirmed in isolated nuclei of stimulated cells. To determine which α-actinin isoform interacts with NF-κB complex, we used A431 cells stably transfected by GFP- α-actinin-1 and GFP- α-actinin-4. Both α-actinin isoforms co-localized with p65, but only α-actinin-4 migrated together with p65 to the nucleus after EGF or TNF-α stimulation. Second, antibodies to different NF-κB subunits co-immunoprecipitated α-actinin-4 from A431 cell lysates and nuclear extracts. Actinin-4 was identified using immunoblotting and MALDI-TOF mass-spectrometry. Third, affinity chromatography indicates that p65 and p50 subunits of NF-κB can bind to matrix-bound chicken gizzard α-actinin. With a blot overlay assay, p65, p50 and IκB-α of cell lysates recognized immobilized α-actinin; and only p65 bound to the 53 kDa fragment of α-actinin. We propose a crucial function of α-actinin-4 isoform in NF-κB nuclear translocation and nuclear events.The work was supported by RFBR grant 03-04-48251 and Visby program of the Swedish Institute. GFP- α-actinin constructs were kindly provided by Dr. Carol Otey.

1261

### Bifurcated Converging Pathways for High Ca++- and TGFb-induced Inhibition of Growth of Normal Human Keratinocytes

M. Sakaguchi,[1] H. Sonegawa,[1] T. Nukui,[1] M. Miyazaki,[1] M. Namba,[2] N. Huh[1]; [1]Cell Biology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan, [2]Niimi College, Okayama, Japan

Growth suppression of normal human keratinocytes by high Ca++ or transforming growth factor b (TGFb) was shown to be mediated by p21WAF1/CIP1 and Sp1. We previously demonstrated that S100C/A11 is a key mediator for growth inhibition of normal human epidermal keratinocytes (NHK) triggered by high Ca++ or TGFb (J. Cell Biol. 163:825-835, 2003; 164:979-984, 2004). On exposure of NHK cells to either agent, S100C/A11 is transferred to nuclei, where it induces p21WAF1/CIP1 through activation of Sp1/Sp3. In the present study, we found that high Ca++ activated NFAT1 through calcineurin-dependent dephosphorylation. In growing NHK cells, KLF16, a member of the Sp/KLF family, bound to the p21WAF1/CIP1 promoter and thereby inhibited the transcription of p21WAF1/CIP1. Sp1 complexed with NFAT1 in high Ca++-treated cells or with Smad3 in TGFb1-treated cells, but not Sp1 alone, replaced KLF16 from the p21WAF1/CIP1 promoter and transcriptionally activated the p21WAF1/CIP1 gene. Thus, high Ca++ and TGFb1 have a common S100C/A11-mediated pathway in addition to a unique pathway (NFAT1-mediated pathway for high Ca++ and Smad-mediated pathway for TGFb1) for exhibiting a growth inhibitory effect on NHK cells, and both pathways were shown to be indispensable for growth inhibition.

1262

### Molecular Chaperones Involved in Vitamin D Receptor Mediated Transcription in Keratinocytes

Y. Oda,[1] N. P. Hawker,[1] S. H. Meijsing,[2] R. J. Chalkley,[3] A. L. Burlingame,[3] D. D. Bikle[1]; [1]Endocrine Research, VA Medical Center San Francisco, San Francisco, CA, [2]Cellular Molecular Pharmacology, University of California San Francisco, San Francisco, CA, [3]Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA

Vitamin D, via its active metabolite 1,25(OH)2D3, controls the proliferation and differentiation of keratinocytes through the vitamin D receptor (VDR) by directly regulating gene transcription. Using GST-VDR affinity beads, VDR binding protein (DRIP) complexes were purified from keratinocytes and identified by mass spectrometry. Within the DRIP complex, we also identified chaperone heat shock proteins (Hsp). Chaperone

proteins are known to maintain the stability of nuclear receptors, although their role in transcription has not been fully elucidated. Recently chaperones have been suggested to be involved in nuclear receptor mediated transcription. Therefore, we evaluated the role of Hsp proteins in VDR stimulated transcription. Direct binding of Hsp 90 and the co-chaperone p23 to the VDRE complex within the 24 hydroxylase and keratin 1 promoters was detected using chromatin immunoprecipitation. When Hsp90 and the co-chaperone p23 were co-localized to a vitamin D response element (VDRE) site using GAL4, they inhibited VDR mediated transcription. Overexpression of p23 promoted removal of VDR from promoter sites. Blockage of chaperone function by geldanamycin induced keratinocyte differentiation shown by increased expression of the early differentiation markers keratin 1 and 10 with no effect on the expression of the basal keratins 5 and 14. These results suggest that chaperone proteins are directly involved in VDR mediated transcription through disassembly of the VDR complex from promoter sites. Inhibition of this function facilitates the transition from a proliferating keratinocyte to a differentiating one.

1263

**The Peptidyl-prolyl Isomerase Pin1 Binds and Regulates the Transcription Factor Nur77**

B. Kim, H. Youn; Biochemistry & Molecular Biology, Seoul National University, Seoul, Republic of Korea

A peptidyl-prolyl isomerase (PPIase), Pin1 specifically recognizes pS(pT)P-motif containing proteins and induces their conformational changes, which ultimately leads to regulating their biological functions. Nur77, an orphan nuclear receptor, plays an important role in apoptosis in immature T cell and cancer cell. Nur77 was originally found as a multiphosphorylated protein. On later, the biological function of Nur77 has been known to be regulated by phosphorylation: DNA-binding ability, transcriptional activity, and translocation from nucleus to cytosol. In this study, we found that Pin1 strongly associates with Nur77 through pS(pT)-P motifs in the N-terminal transactivation domain(N-TAD) of Nur77. In particular, the mutations of three serine residues (S196, S202, S240) in N-TAD completely abrogated the Pin1-Nur77 interaction, indicating that Pin1 specifically binds to Nur77 through pS-P motifs in N-TAD. Ectopically expressing Pin1 decreased Nur77 transcriptional activity. Moreover, a Nur77 mutant [Nur77(S196A/S202A/S240A)] that cannot interact with Pin1 has a higher transcriptional activity than Nur77(WT). Taken together, we suggest that Pin1 may negatively regulate Nur77 transcriptional activity under the certain conditions that Nur77 is phosphorylated at three pS-P motifs.

1264

**A Novel Krüppel Related Factor Consisting of Only a KRAB-A Domain is Expressed in the Murine Trigeminal Ganglion**

K. Nikulina, M. Bodeker, J. Warren, A. Ellison, T. P. Margolis; F. I. Proctor Foundation, University of California San Francisco, San Francisco, CA

Productive infection with herpes simplex virus (HSV) type 1 requires activation of the promoter of the viral IE110 gene, which codes for the protein ICP4. A functional screen for possible repressors of the IE110 promoter led to the cloning of a rat gene, If1, encoding a Krüppel -associated box-A (KRAB-A) protein, whose expression had an inhibitory effect on HSV replication in vitro. Screening a murine cDNA library with a probe based on the If1 sequence resulted in the isolation of a mouse If1 homologue (Mif1) encoding a small 8.2 kDA protein comprised largely of a single KRAB-A domain. Northern and Western blot analysis of extracts of murine trigeminal ganglion revealed KRAB-A containing transcripts about 600 bps in size and small KRAB-A containing proteins. These data demonstrate the existence of a subset of Krüppel related factors which are comprised of only a KRAB-A box and no additional downstream domains, such as zinc fingers, normally seen in typical KRAB-domain proteins. Yeast two-hybrid analysis showed that Mif1 interacts with co-repressor TIF1β suggesting the same mechanism of regulation as typical KRAB-domain proteins. In a GAL4 fusion repressor assay Mif1 demonstrated significant downregulation of the herpes IE110 promoter. These results contribute to the discussion of the evolutionary and functional relationship of non-zinc finger KRAB-proteins and typical Krüppel zinc finger genes and there role and relevance to viral infection.

1265

**Translocation of Membrane EGF Receptor to Nucleus: Function and Mechanism**

M. Hung, S. Wang, H. Lo, S. Hsu, W. Xia, Y. Wei; Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX

Previously, we have found that the rat neu, a member of epidermal growth factor receptor (EGFR) family, was localized in the nucleus and could act as a transcriptional activator. Later, we demonstrated that the carboxyl terminus of EGFR contains a strong transactivation activity and EGFR complex recognizes an AT-rich consensus-sequence on cyclin D1 promoter. By using ChIP assays, we showed that nuclear EGFR associated with promoter region of cyclin D1 *in vivo*. EGFR may function as a transcription factor in the nucleus. Recently, using the ChIP cloning strategy, we identify that human HER-2/ ErbB-2 was associated with multiple genomic targets *in vivo*, including the *COX-2* gene promoter. We have shown that ErbB-2 forms a complex at a specific nucleotide sequence of the *COX-2* promoter and is able to stimulate its transcription. This study strengthens the functionality of ErbB-2 as a transcriptional regulator in the nucleus. Moreover, we demonstrate that EGFR physically interacts with signal transducers and activators of transcription 3 (STAT3) in the nucleus leading to transcriptional activation of inducible nitric oxide synthase (iNOS). In breast carcinomas, nuclear EGFR positively correlates with iNOS expression. The study describes a mode of transcriptional control involving cooperated efforts of STAT3 and nuclear EGFR. Our work suggests de-regulated iNOS/NO pathway may partly contribute to the malignant biology of tumor cells with high levels of nuclear EGFR and STAT3. In addition, we have analyzed 130 breast carcinomas via immunohistochemical analyses for the nuclear and non-nuclear EGFR. Kaplan-Meier survival analysis and log-rank test revealed a significant inverse correlation between high nuclear EGFR and overall survival ($P = 0.009$). Thus, our findings indicate pathological significance of nuclear EGFR and may have important clinical implication.

1266

**Phosphorylation of Ser1834 by Akt Enhances the HAT and Transcriptional Activities of p300**

C. Chen, W. Huang; Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan Republic of China

The PI3K/Akt pathway plays a critical role in the regulation of gene expression induced by numerous stimuli. p300, a transcriptional co-activator, acts in concert with transcription factors to facilitate gene expressions. Here we showed that Akt is activated and translocated to the nucleus in response to TNF-α. Nuclear Akt associates with p300 and phosphorylates its Ser-1834 both *in vivo* and *in vitro.* The phosphorylation induces

recruitment of p300 to the *ICAM-1* promoter, leading to the acetylation of histones in chromatin and association with basal transcriptional machinery RNA polymerase II. These two events, abolished by the p300 S1834A mutant, inhibitors of PI3K/Akt or siRNA of Akt, facilitate the ICAM-1 gene expression. Histone acetylation is attributed to the Akt-enhanced intrinsic HAT activity of p300 and its association with other HAT, p/CAF. Our study provides a new insight into the molecular mechanism by which Akt promotes the transcriptional potential of p300.

1267

**Nuclear Speckle-associated Protein Pnn/DRS Forms a Complex With RNA Polymerase II and Impacts E-cadherin mRNA Processing**

R. Alpatov, G. C. Munguba, S. P. Sugrue; Anatomy and Cell Biology, University of Florida, Gainesville, FL

Pnn/DRS (Pnn) is a "nuclear speckle"-associated multifunctional protein known to participate in mRNA processing in the nucleus. It is also directly involved in transcriptional regulation of the E-cadherin gene via its binding to CtBP, a multifunctional transcriptional repressor that targets the E-cadherin promoter. In light of recent reports of Pnn's presence in complexes associated with basal transcriptional machinery, we wished to address the possible interaction of Pnn with RNA polymerase II, which might imply Pnn-dependent coordination of transcription and mRNA splicing events. *In vivo* binding assays, using HeLa nuclear extracts, showed that endogenous Pnn and Pol II can interact. In addition, we demonstrated that Pnn preferentially associates with the transcriptionally active forms of Pol II: initiation-specific Pol II (phosphorylated on Ser5), elongation-specific Pol II (phosphorylated on Ser2), but not with pre-initiation Pol II (unphosphorylated). Immunofluoresence studies using NIH3T3hPnnGFP cells, which stably express epitope-tagged Pnn also demonstrated higher degree of overlap between Pnn and transcriptionally active forms of Pol as compared to pre-initiation Pol II. In addition, as an indication of their potential functional coupling, Pnn and active forms of Pol II exhibited similar redistribution dynamics in response to the actinomycin D-mediated transcriptional block, accumulating as enlarged, round speckles. Furthermore, using splicing reporter assay, utilizing E-cadherin exon4-intron-exon5 cassette driven by E-cadherin or SV-40 promoter, we demonstrated that Pnn can positively impact E-cadherin mRNA splicing efficiency in the promoter-specific manner. These data contribute to our understanding of Pnn's role in regulation of transcription as a coupling factor that can bridge components of basal transcriptional machinery and mRNA processing events. Supported by NIH grant EY07883

1268

**Role of Phospholipase C and Inositol Polyphosphates in Transcriptional Regulation in Budding Yeast**

A. Demczuk, N. Guha, J. Chang, A. Vancura; Biological Sciences, St. John's University, Jamaica, NY

Phosphatidylinositol phosphates (PtdInsPs) are involved in signal transduction, cytoskeletal organization, and membrane trafficking. Inositol polyphosphates (InsPs), produced from PtdInsPs by the phospholipase C-dependent pathway, regulate chromatin remodeling. We used genome-wide expression analysis to further investigate the role of Plc1p (phosphoinositide-specific phospholipase C) and InsPs in transcriptional regulation. Plc1p regulates the expression of approximately 3% of yeast genes in cells grown in rich medium. Most of the genes are repressed by Plc1p. Surprisingly, genes regulated by Plc1p do not correlate with gene sets regulated by Swi/Snf or RSC chromatin remodeling complexes. In addition, the patterns of genetic interactions displayed by *plc1*Δ and *swi2*Δ mutations are distinct, however partly overlapping. Genes repressed by Plc1p show correlation with genes induced by diauxic shift and gene sets controlled by Msn2p/Msn4p, Srb10p, Mot1p, and NC2. The Plc1p-dependent repression of Srb10p-regulated genes does not involve regulation of Srb10p level or activity by Plc1p. Plc1p does not regulate nucleocytoplasmic distribution of Msn2p; however, *plc1*Δ cells display increased recruitment of Msn2p to the promoters of Msn2p-regulated genes. Taken together, the results indicate that in addition to chromatin remodeling, InsPs produced by the Plc1p-dependent pathway affect transcriptional regulation by yet another mechanism, possibly involving recruitment of Msn2p/Msn4p and formation of the preinitiation complex.

1269

**Expression of ABC Transporter *FLR1* Requires Phospholipase C and is Repressed by Mediator**

P. Desai, C. Romero; Biological Sciences, St. John's University, Jamaica, NY

In budding yeast, phosphoinositide - specific phospholipase C (Plc1p encoded by *PLC1* gene) is important for function of kinetochores. Deletion of *PLC1* results in benomyl sensitivity, alterations in chromatin structure of centromeres, mitotic delay, and a higher frequency of chromosome loss. In this study, we intended to utilize benomyl sensitivity as a phenotype that would allow us to identify genes that are important for kinetochore function and are downstream of Plc1p. However, our screen identified *SIN4*, encoding a component of the Mediator complex of RNA polymerase II. Deletion of *SIN4* gene does not suppress benomyl sensitivity of *plc1*Δ cells by improving the function of kinetochores. Instead, benomyl sensitivity of *plc1*Δ cells is caused by a defect in expression of *FLR1* and the suppression of benomyl sensitivity in *plc1*Δ *sin4*Δ cells occurs by derepression of *FLR1* transcription. *FLR1* encodes a plasma membrane transporter that mediates resistance to benomyl. Several other mutations in the Mediator complex also result in significant derepression of *FLR1* and greatly increased resistance to benomyl. Thus, benomyl sensitivity is not a phenotype exclusively associated with mitotic spindle defect. These results demonstrate that in addition to promoter-specific transcription factors that are components of the pleiotropic drug resistance network, expression of the membrane transporters can be regulated by Plc1p, a component of a signal transduction pathway, and by Mediator, a general transcription factor. The results thus suggest another layer of complexity in regulation of the pleiotropic drug resistance.

1270

**Elucidating the Function of the Truncated Hoxa1 Protein**

C. C. Fernandez, L. J. Gudas; Department of Pharmacology, Weill Medical College of Cornell University, New York, NY

Retinoic acid (RA), the natural acidic metabolite of vitamin A, has been shown to influence the proliferation and differentiation of a wide variety of different cell types. More specifically, RA plays an important role in cellular differentiation by transcriptionally up-regulating the expression of *Hox* genes. *Hox* genes are involved in establishing anterior to posterior pattern formation during vertebrate development. They act as transcription factors that characteristically bind to DNA through a helix-turn-helix motif termed the homeodomain. Previously, our lab has shown that RA transcriptionally regulates the expression of *Hoxa1* in F9 teratocarcinoma cells. The *Hoxa1* gene encodes two alternatively spliced mRNAs that can direct the synthesis of two distinct proteins, one that contains the homeobox, Hoxa1-993, and another truncated protein that lacks the DNA binding domain, Hoxa1-399. The function of the truncated protein is not known. I have used both wild type F9 and EW-F9 mutant embryonic stem cells,

which overexpress the Hoxa1-399 transcript in the presence of zinc, to examine the effects of the shorter Hoxa1 protein on the expression of Hoxa1 target genes. Overexpression of Hoxa1-399 in EW-F9 cells enhances the expression of endogenous *Hoxa1*, *Hoxb1* (a known Hoxa1 target), and various Hox cofactors. Results of GST pull-down assays show that Hoxa1-399 and Hoxa1-993 dimerize *in vitro*. Therefore, these results indicate that one function of the truncated Hoxa-1 protein is to up-regulate the expression of Hoxa-1 target genes. However, reporter assays using a small region of the *Hoxb1* promoter to drive luciferase expression implicate Hoxa1-399 as a negative regulator of the transcriptional activation of the Hoxa1 target gene, *Hoxb1.*

1272

**Modulation of Human SIM1 and SIM2 Transcription Factors**

Y. Shimizu,[1] A. Yamaki,[1] M. Okui,[2] A. Takayanagi,[2] J. Kudoh,[2] N. Shimizu[2]; [1]Medical Genetics, Kyorin University School of Health Sciences, Hachioji-shi Tokyo, Japan, [2]Molecular Biology, Keio University School of Medicine, Shinjuku-ku Tokyo, Japan

Human SIM1 and SIM2 proteins are members of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors. Previously, we identified the nuclear localization signal (NLS) at the aa 367-388 region of SIM1 and the aa 367-388 region of SIM2 (Biochem. Biophys. Res. Commun. 313:482-488, 2004). Here, we examined the modulation of the cellular localization of SIM1 and SIM2 proteins by ARNT or ARNT2. The NLS deficient mutants of SIM1 or SIM2 remain in the cytoplasm but cotransfection with ARNT or ARNT2 resulted in the nuclear localization of these SIM mutants, indicating the direct association of SIM and ARNT proteins. Interestingly, we found that SIM1 and SIM2 were poly-ubiquitinated in transiently transfected cells, and the poly-ubiquitinated proteins were more accumulated in the presence of a proteasome inhibitor MG132 in the stable transfectants. Furthermore, SIM1 and SIM2 were immunoprecipitated with the RING-IBR-RING-type E3 ubiquitin ligases, such as Parkin and HHAR1, but they were not immunoprecipitated with other E3 ligases, such as one RING type Siah-1 and the PHD type AIRE. Using the deletion constructs and mutants of SIM2, the poly-ubiquitination sites were identified at multiple Lys residues in the aa141-289 region. Poly-ubiquitinated SIM1 and SIM2 were immunoprecipitated with ARNT or ARNT2. These findings indicate that SIM1 and SIM2, form a complex with ARNT and their function may be regulated through poly-ubiquitination during the brain development.

1273

**Multiple Mechanisms Regulate Methionine Biosynthetic Gene Network Transcription in the Yeast *Saccharomyces cerevisiae***

S. Chandrasekaran,[1] A. Deffenbaugh,[2] D. Skowyra,[2] N. Mathias[1]; [1]Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA, [2]Biochemistry and Molecular Biology, St. Louis University, St. Louis, MO

When depleted of sulfur-containing amino acids, yeast cells remodel their transcription profile allowing expression of the methionine biosynthetic (*MET*) gene network. This network encodes transporters and enzymes required for the uptake and assimilation of extra-cellular sulfate to methionine, S-adenosyl methionine, and cysteine. A single transcription activator, Met4p, mediates the expression of the entire *MET* network and binds *MET* promoters through its association with four auxiliary proteins. Met4p activity is regulated by a negative feedback mechanism; however, different *MET* genes are repressed to different extents. Our objective is to understand how the abundance of methionine and cysteine are sensed and the mechanisms used to regulate Met4p activity. The $SCF^{Met30p}$ ubiquitin ligase is critical for *MET* gene repression. We have shown that overproduction of Met30p (the substrate recognition component of the ligase) increases the poly-ubiquitinated state of Met4p and represses *MET* gene expression. Thus, poly-ubiquitination of Met4p is sufficient to reduce its activity. By reconstituting the ubiquitination and proteasome-dependent degradation of Met4p, we have revealed that Met4p may be ubiquitinated in both - met and + met growth conditions but is only degraded in + met conditions. Thus, proteolysis of Met4p is important for repressing *MET* gene activity. The rate-limiting step of Met4p proteolysis is its release from its auxiliary proteins, which can be stimulated by the addition of exogenous cysteine and methionine. Thus, these amino acids evict Met4p from its transcriptional complexes and likely do so directly. Hence, we propose that multiple mechanisms regulate Met4p including its ubiquitination, interaction with auxiliary proteins, and degradation. Furthermore, we speculate that these mechanisms are not equally employed at each *MET* promoter, explaining how different *MET* genes are repressed to different extents.

1274

**Silencing of Retinoic Acid Receptors: An Epigenetic Domino Effect?**

S. Pozzi, G. Bistulfi, S. Rossetti, R. MingQiang, N. Sacchi; Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY

Resistance to the growth inhibitory action of retinoic acid (RA), the bioactive derivative of Vitamin A is common in human tumors. One form of RA resistance has been associated with aberrant DNA methylation and silencing of the retinoic acid receptor beta 2 (*RARβ2*) gene, whose transcription is regulated by RA. Understanding why *RARβ2* is prone to aberrant epigenetic silencing in human cells is critical for both prevention and early treatment of RA-resistance in human carcinoma cells. Here we show that an exacerbation of *RARβ2* transcriptional inactivity in human cells leads to heritable *RARβ2* DNA methylation and silencing. Further, we show that *RARβ2* epigenetic silencing leads to the development of RA-resistance and distinct features of tumor progression. This study serves as the proof of principle that epigenetic silencing can be consequent to an exacerbated transcriptional inactivity. Notably, *RARβ2* silencing is related to the silencing of other nuclear receptors.

1275

**Activation of the PI3K/Akt (PKB)/Bad Cascade by 1α,25-dihydroxy Vitamin $D_3$ in Osteoblasts**

X. Zhang, L. P. Zanello; Biochemistry, University of California-Riverside, Riverside, CA

1α,25-dihydroxy vitamin $D_3$ [1,25D], the biologically most active form of vitamin D, elicits cellular responses by means of two different mechanisms. On one hand, a genomic pathway involves regulation of gene transcription via a nuclear vitamin D receptor. On the other, a non-genomic pathway, which initiates at the plasma membrane level, comprises activation of specific cytoplasmic kinase cascades in different cell types. In osteoblasts, 1,25D promotes bone anabolic actions through both genomic and non-genomic pathways. However, the precise molecular mechanisms of membrane-initiated 1,25D actions are only partially understood. We report here 1,25D-activation of Akt phosphorylation in osteoblastic ROS 17/2.8 cells. Akt (protein kinase B), a PI3K downstream molecule, has been implicated in several cellular responses, including cell survival. Using a specific anti-phospho-serine 473 Akt antibody, we detected a dose dependent significant increase in Akt phosphorylation by 1,25D, with maximal response achieved with 10 nM of hormone. Time course experiments showed that hormone-stimulated Akt phosphorylation

can be measured as early as 5 min after treatment, and reached its maximum at around 10 min. In addition, we found that 1,25D induces phosphorylation of Bad, a downstream molecule of Akt implicated in cell survival. We show here that 10 nM 1,25D significantly reduces staurosporine-induced apoptosis in ROS17/2.8. Apoptotic cells were detected with propidium iodide stain and flow cytometry analysis. We conclude that rapid responses to 1,25D leading to cell survival occur via the PI3K/Akt/Bad pathway in osteoblasts. Anti-apoptotic effects of 1,25D may contribute to enhanced bone formation by osteoblasts treated with the steroid hormone. [Funded by NIH DK071115-01]

1276

**Gene Expression Profiling of the Estrogen Responsive Genes by the Treatment With Alkylphenols, Chlorinated Phenols, Parabens, or Bis- and Benzoylphenols for Evaluation of Estrogen Activity**

M. Tanji,[1] S. Terasaka,[2,1] A. Inoue,[1] R. Kiyama[3,1]; [1]InfoGenes Co., Ltd., Tsukuba, Ibaraki, Japan, [2]SciMedia Ltd., Taito-ku, Tokyo, Japan, [3]National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan

We examined expression profiles of the estrogen responsive genes after the treatment with alkylphenols (p-cresol, 4-n-ethylphenol, 4-n-heptylphenol, 4-t-octylphenol and nonylphenol), chlorinated phenols (4-chlorophenol, 4-chloro-3,5-dimethylphenol, 2,4-dichlorophenol and pentachlorophenol), parabens (methylparaben, ethylparaben propylparaben and butylparaben), or bis- and benzoylphenols (bisphenols A and B, and p-hydroxybenzophenone) by means of DNA microarray assay for evaluation of estrogen activity of these chemicals. By selecting a set of 120 genes showing greater statistical reliability for estrogen, a greater reliability of the assays for the chemicals shown above was achieved and, for the chemicals whose data were available, the results were consistent with those by the previously reported ER-binding and yeast two-hybrid assays. Evaluation of the chemicals by gene functions indicated that the genes related to proliferation, transcription and transport were mostly up-regulated while significant numbers of genes related to enzymes and signaling showed down-regulation. The genes related to transcription showed the highest degree of variations among six categorized gene functions for the chemicals with relatively high estrogen activities. These results indicate that the variations of chemicals and their biological effects can be monitored by appropriate grouping of the estrogen responsive genes.

1277

**Protein Phosphatase 2A (PP2A) Transfer onto the Androgen Receptor (AR) Requires Specific HEAT Repeats in PP2A and the Agonist Conformation of AR**

C. Yang,[1] D. L. Brautigan,[2] B. M. Paschal[3]; [1]Center for Cell Signaling, University of Virginia, Charlottesville, VA, [2]Center for Cell Signaling, Department of Microbiology, University of Virginia, Charlottesville, VA, [3]Center for Cell Signaling, Department of Biochemistry & Molecular Genetics, University of Virginia, Charlottesville, VA

Nuclear receptor transcriptional activity is regulated by mechanisms including ligand binding and phosphorylation. In the course of characterizing phospho-regulation of AR, we found that SV40 small t antigen (ST) mediates selective transfer of the PP2A A/C heterodimer onto AR (MCB 25: 1298, 2005). Using deletion analysis, protein-interaction assays, and the published crystal structure of PP2A, we determined that AR contacts HEAT repeat 14 in the A-subunit of PP2A. Since ST binding is centered on HEAT repeat 4, ST apparently causes a conformational change that is transduced ~76 angstroms to HEAT repeat 14. Induced-conformation represents a novel form of PP2A targeting, which normally relies on a B-subunit to target PP2A to its substrates. Because PP2A binds exclusively to the agonist-bound form of AR, we hypothesized that PP2A dissociation would be driven by androgen dissociation. We demonstrated this by correlating androgen and PP2A dissociation rates in vivo (R2=0.9859). Non-androgenic compounds can activate AR and may explain how prostate cancer cells escape androgen deprivation therapy. We tested a variety of compounds (androgen agonists, antagonists, and estradiol) to determine whether non-androgenic compounds can induce a conformation of AR that is suitable for accepting PP2A. Only R1881, dihydrotestosterone and the adrenal androgen androstenedione induced an AR conformation that bound PP2A in the ST transfer reaction. Because non-androgenic compounds induce an AR conformation that is functional for transcription but not for PP2A transfer, we conclude that at least two different conformations of AR can promote transcription. The PP2A-bound form of AR binds androgen response element DNA in gel shift assays, leading us to suggest that AR might target PP2A to the promoters of androgen-regulated genes.

1278

**Progesterone and Estrogen Receptor Profiles in the Human Corneal and Conjunctival Tissues, Cells and Cell Lines**

J. Shankardas,[1] V. V. Mootha,[2] S. D. Dimitrijevich[1]; [1]Integrative Physiology, UNT Health Science Center, Fort Worth, TX, [2]Ophthalmology, UT Southwestern Medical School, Dallas, TX

**Background**: There is increasing evidence for the role of age and gender in some pathologies. Thus dry eye syndromes and Fuchs endothelial dystrophy are prevalent amongst post-menopausal women. The effect of changing hormonal levels on the human anterior ocular compartment requires a clear profile of the relevant hormone receptors in these tissues and their cellular components. **Purpose**: The objectives of this study were to determine: a. the profile of estrogen and progesterone receptors in the corneal and conjunctival tissue, b. the profile of these receptors in the primary keratocytes, epithelial and endothelial cells c. the presence of these receptors in the epithelial and endothelial cell lines. Method: The lysates of the corneal and conjunctival epithelia were obtained after isolation from donor tissue with dispase. The primary cells were subcultured on glass coverslips, fixed-permeabilized and examined by indirect immunoflurescence. The hTERT transformed epithelial cells and E6/E7 transformed endothelial cells were also examined by indirect immunofluorescence. The deparaffinized cross-sections of the donor corneas and conjunctivas were examined by indirect immunofluorescence. The receptor profiles were confirmed by western analysis of cell and tissue lysates. **Results**: Indirect immunofluorescence showed a variable intracellular localization of the estrogen receptors (ERa, ERb) in all the cells, cell lines and tissues from male donors. The presence of progesterone receptors (PRA, PRB) was only detected in the corneal epithelial cells, the cell line, the endothelial cells, and the corneal sections. The western analysis confirmed the presence of PRA and PRB and ERa and ERb. **Conclusions**: Indirect immunofluorescence and western blot analysis show the presence of progesterone (A and B) and estrogen (a, b) receptors in the cellular components of the cornea and conjunctiva, and tissue cross- sections from male donors.

## Chromatin & Chromosomes I (1279-1297)

1279

### Instability of Foreign DNA Integrated into the Genome of Transformed Cells

A. V. Ivanov; Cell Biology Laboratory, Petersburg Nuclear Physics Institute RAS, Gatchina, Russian Federation

This study describes the faith of foreign DNA integrated into the cultured mammalian cell genome. The apposite model system was created. It contains in Chinese hamster cell line A23 TK-, a plasmid vector p16 with two selective genes NEO (G418 resistance) and TK (thymidinkinase) of human herpes simple virus type 1 (HSV). Presence of TK HSV makes cells sensible to the herpes drug ganciclovir and resistant for HAT medium (hypoxanthine, aminoptherine, thymidine). The collection of 64 independent clones was created. Four types of transgene behavior are found reflecting different rates of plasmid DNA instability. The real deleting of transfected DNA from genome was demonstrated using the PCR technology. This data fully check with the selection features losses pattern. In majority it is seems the both integrated plasmid selective genes are lost, but in several cases just one gene is lost and another is steel present. The possibility of homological recombination increase by means RecA - like bacterial recombinases is tested. To check the supposition of high instability sites near integrated foreign DNA presence into the genome (has been existed or formatted during the integrative process) the work of cloning flank sequences of exogenic DNA was carried out. 10 k.b.p. plasmid containing both the fragments of primary plasmid p16 and sequences flanking regions of integration to the cell genome was cloned. Transfection of this plasmid to A23 cells made out resulting of 100 % unstable integration in 18 clones. Such data confirm the presence of defensive mechanisms, which prevent integration and preservation of exogene DNA in the eukaryotic cell genome. Main suggestions of molecular mechanisms are homologous recombination and integration to freak sites of chromosomes.

1280

### Ultrastructure of Euchromatin Contact Points between the Closed Loops of Adjacent Interphase Chromosomes

J. H. Frenster, J. A. Hovsepian; RNA Research, Physicians' Educational Series, Atherton, CA

Recent fluorescence microscopy studies (Parada LA, McQueen PG, and Misteli T, Genome Biology, vol. 5, no. 7, r44 (June 21, 2004), involving a systematic analysis of the spatial positioning of a subset of mouse chromosomes in several tissues, have suggested the possibility of an associative clustering of particular chromosomes in particular tissues during nuclear interphase. We have noted a similar phenomenon in isolated lymphocyte nuclei of calf thymus (Frenster JH, Nature 205: 1341 (1965), and have further analyzed our electron micrographs in order to define the ultrastructure of the phenomena. In the course of the isolation of repressed and active chromatin from interphase calf thymus lymphocytes, a stage is reached in which the nuclei swell to twice their normal size, offering a favorable material for the examination of the structural relations between the active euchromatin microfibrils and the repressed heterochromatin masses. Such swollen nuclei were gently prepared in cation-free isotonic sucrose, and examined for the location, number, shape, caliber, and length of continuous, closed loops of the 10 nm caliber active euchromatin microfibrils. The closed loops appear to be tethered at intervals of 50-100 nm to single masses of repressed heterochromatin chromocenters. The loops extend out into the interior of the nucleus for up to 1 um, forming elongated lariats that contact similar loops from adjacent chromocenters. At the point of close apposition between opposing loops from distinct chromocenters, dense microcylinders of 20-30 nm caliber are formed, appearing to represent equal contributions from each chromocenter. The microcylinders range up to 100 nm in length before separating into their original calibers of 10 nm each. The fields of such chromosomal apposition range up to 3 um in diameter, and are often elliptical in outline.

1281

### Probing the Chromatin Assembly Factor 1 Proteome

M. Hoek, M. Myers, B. Stillman; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

The chromatin assembly complex CAF-1 couples chromatin assembly to DNA replication and is essential for passage through S-phase in human cell lines. Recent evidence also suggests that CAF-1 may coordinate other activities on chromatin in a replication and DNA repair dependent manner. To gain insight into the multiple functions of CAF-1, we have begun to systematically analyze the set of CAF-1 interacting proteins. Cell lines expressing a FLAG-HA tagged p150 subunit of CAF-1 were established in RKO cells and the tagged protein was shown to be functional by RNAi rescue experiments. When the tagged protein complex was purified under standard conditions from nuclear extracts few novel CAF-1 interacting proteins were identified. However, when the stringency of the immunoprecipitation conditions was reduced, many more putative CAF-1 interacting proteins as well as previously identified CAF-1 partners such as PCNA and HP1 were recovered. cDNAs encoding these proteins were cloned and screened for interaction with recombinant CAF-1 using in vitro translation. To date, one strong interaction with the Ku86/Ku70 complex and several weaker interactions with other proteins have been recapitulated in this secondary screen. The interaction between CAF-1 and Ku requires both subunits of the Ku complex, emphasizing the utility of this approach in identifying interactions that are not binary. Protein phosphatase inhibitors and ATP are both required for the stable interaction between these complexes, suggesting that phosphorylation of one or both components is required. We are currently attempting to identify kinases important in regulating this interaction and are testing whether CAF-1 may be recruited to double-strand breaks that are bound by Ku.

1282

### A Temperature-Sensitive Mutation in the WD Repeat-Containing Protein Smu1 Is Related to Maintenance of Chromosome Integrity

K. Sugaya,[1] E. Hongo,[1] Y. Ishihara,[1] H. Tsuji[2]; [1]Radiation Hazards Research Group, National Institute of Radiological Sciences, Chiba, Japan, [2]Low Dose Radiation Effects Research Project, National Institute of Radiological Sciences, Chiba, Japan

Temperature-sensitive CHO-K1 mutant cell line tsTM18 exhibits chromosomal instability and cell cycle arrest at S and $G_2$ phases with decreased DNA synthesis at the nonpermissive temperature, 39°C. To identify the causative mutation, we fused tsTM18 cells with normal human cells to generate hybrids carrying fragments of human chromosomes. Analysis of chromosome content of temperature-resistant transformants and introduction of a bacterial artificial chromosome containing part of human chromosome 9 led to isolation of the human *SMU1* gene. Comparison of sequences of the *Smu1* gene from wild-type and mutant cells revealed that the mutant phenotype is caused by a G-to-A transition that yields a gly-to-arg substitution at position 489 in hamster Smu1. The substituted glycine is located in the WD-repeat domain of Smu1. Single-stranded DNA accumulated in the nuclei of mutant cells at 39°C. Furthermore, cdc2 kinase was not activated during $G_2$ phase, and there was no chromosome

segregation due to incomplete assembly of the spindle during M phase. Thus, Smu1 appears to be involved directly or indirectly in DNA replication, activation of cdc2 kinase, spindle assembly, and maintenance of chromosome integrity, reflecting the important roles of Smu1 in cellular function.

1283

**Changes in Chromatin Structure and Mobility in Living Cells at Sites of DNA Double-strand Breaks**

M. J. Kruhlak,[1] A. Celeste,[1] O. Fernandez-Capetillo,[1] G. Dellaire,[2] W. Muller,[1] J. G. McNally,[1] D. P. Bazett-Jones,[2] A. Nussenzweig[1]; [1]National Cancer Institute, National Institutes of Health, Bethesda, MD, [2]Programme in Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada

The repair of DNA double strand breaks (DSBs) is facilitated by the phosphorylation of H2AX which organizes DNA damage signaling and chromatin-remodeling complexes in the vicinity of the lesion. Large-scale relaxation of chromatin has been proposed to activate the DNA damage transducer ATM at a distance, and may also increase the mobility of DSB-containing chromatin domains, whereas damage-mediated condensation may facilitate faithful repair by limiting the diffusion of broken ends. Although indirect evidence supports the idea that disruption of DNA integrity induces an alteration of chromatin architecture, little is known about the physical properties of damaged chromatin. Here we use a photoactivatable version of GFP-tagged histone H2B to examine the mobility and structure of chromatin containing experimentally induced DSBs in living cells. We find that bulk chromatin containing DSBs exhibits limited mobility but undergoes an energy-dependent local expansion immediately following DNA damage. To determine the ultrastructure of irradiation induced foci (IRIF) and the chromatin changes associated with the activation of ATM, we employed a correlative fluorescence and energy-filtering transmission electron microscopy imaging method. We find that the localized expansion observed in real-time constitutes a relaxation in higher order chromatin structure, corresponding to a 30-40% reduction in the density of chromatin fibers in the vicinity of DSBs. The observed opening of chromatin occurs independently of H2AX and restricts the initial activation of ATM to the locally damaged region. We propose that localized ATP dependent decondensation of chromatin at DSBs increases the accessibility of regions of damaged DNA to protein complexes, thereby establishing a sub-nuclear environment that facilitates DNA damage signaling and repair.

1284

**Calcium-Mediated Proteolytic Processing and Cellular Locations of DNA Topoisomerases**

S. Chou, H. Chen, T. Huang, T. Li; Department and Graduate Institute of Microbiology, Scholl of Medicine, National Taiwan University, Taipei, Taiwan Republic of China

DNA topoisomerases perform their functions by catalyzing the transient breakage and rejoining of DNA strands. Substantial evidences have shown that many cations participate in the catalytic process and/or the regulation of enzymatic activity of this class of enzymes. Here we observed that, within 15min of treatment, calcium ($Ca^{2+}$) ionophores, ionomycin and A23187, efficiently triggered proteolytic cleavage of human DNA topoisomerase I (hTOP1) and topoisomerase II beta (hTOP2β), but not hTOP2α. The ionomycin-induced proteolytic processing of topoisomerases can be blocked by pre- or co-treatment of $Ca^{2+}$ chelators supports the involvement of $Ca^{2+}$ in this process. In addition, addition of $Ca^{2+}$ into culture medium can also efficiently activate the proteolytic processing of DNA topoisomerases with identical patterns to those exhibited by cells treated with ionomycin. The caspase (Z-VAD-fmk) and 26S proteasome (MG132, lactacystin) inhibitors were found to have only minimal effects on $Ca^{2+}$-activated proteolytic events. Nevertheless, pretreatment of HCT116 cells with calpain inhibitor I or II can efficiently repress the $Ca^{2+}$-activated processing of topoisomerases. Thus, the calpain proteases are most likely to be critically involved in the ionomycin-induced, $Ca^{2+}$-activated proteolytic processing of DNA topoisomerases. In agreement with this notion, transient expression of calpastatin, a natural calpain inhibitor, can reduce the ability of ionomycin to activate the proteolytic processing of topoisomerases. Based on the epitope recognitions of the antibodies used, we conclude that both cleavages of DNA TOP1 and TOP2β occur on C'-terminus. The biological implication of our discovery remains unclear. However, the nuclear distributions of hTOP1 and hTOP2β are delocalized and further concentrated in the nucleolus upon ionomycin treatment, possibly through regulating the SUMOylation status of topoisomerases. The relationship between $Ca^{2+}$, SUMOylation states and cellular locations of DNA topoisomerases are discussed.

1285

**Condensin I is Essential for the Structural Integrity of Centromeric Heterochromatin During Mitosis**

R. A. Oliveira, P. A. Coelho, C. E. Sunkel; Molecular Genetics of Mitosis, Institute for Molecular and Cell Biology, Porto, Portugal

During cell division chromatin undergoes structural changes essential to ensure faithful segregation of the genome. Condensins are abundant components of mitotic chromosomes and are known to form two different complexes, condensin I and II. To further examine the role of condensin I in chromosome structure and in particular in centromere organization, we depleted from S2 cells the *Drosophila* CAP-H homologue (Barren), a subunit exclusively associated with condensin I. Immunofluorescence and *in vivo* analysis of Barren/CAP-H depleted cells showed that mitotic chromosomes are able to condense but fail to resolve sister chromatids. Additionally, Barren/CAP-H depleted cells show chromosome congression defects that do not appear to be due to abnormal kinetochore-microtubule interaction. Instead, the centromeric and pericentromeric heterochromatin of Barren/CAP-H depleted chromosomes shows structural problems. After bipolar attachment, the centromeric heterochromatin organized in the absence of Barren/CAP-H cannot withstand the forces exerted by the mitotic spindle and undergoes irreversible distortion. Taken together, our data suggests that the condensin I complex is required not only to promote sister chromatid resolution but also to maintain the structural integrity of centromeric heterochromatin during mitosis.

1286

**Long-range Directional Movement of Interphase Chromosome Site Dependent on Actin and Nuclear Myosin**

C. Chuang; Dept. of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL

Increasing evidence suggests functional compartmentalization of interphase nuclei. This includes preferential interior localization of gene rich and early replicating chromosome regions versus peripheral localization of gene poor and late replicating chromosome regions, association of some active genes with nuclear speckles, and association of transcriptionally repressed genes with heterochromatic regions. How is this differential chromosome positioning established? Does interphase chromosome motion occur passively, through regulating attachment and detachment from nuclear substructures but with random diffusion between nuclear compartments, or is it actively driven, through long-range, directed motion? Here

we demonstrate migration of an interphase, mammalian chromosome site from the nuclear periphery to interior 1-2 hours after targeting a transcriptional activator to this site. Migration is perturbed by specific actin or nuclear myosin I mutants. Extended periods of chromosome immobility are interspersed with several minute periods in which chromosomes move unidirectionally along curvilinear paths oriented roughly perpendicular to the nuclear envelope at velocities of 0.1-0.9 mm/min over distances of 1-5 mm. Our results reveal an active mechanism for fast and directed long-range interphase chromosome movements.

1287

**Visualization of Chromosome Structure and Localization of Chromosomal Proteins in Human Metaphase Chromosomes**

L. Lin,[1] M. Yanagisawa,[1] S. Uchiyama,[1] S. Inaga,[2] S. Matsunaga,[1] A. Takaoka,[1] K. Fukui[1]; [1]Osaka University, Suita, Japan, [2]Tottri University, Yonago, Japan

Genomic DNA of higher eukaryote highly condenses and forms chromosomes during mitosis. Chromosome condensation is indispensable for precise and homogeneous segregation of daughter cells. The model of chromosome organization has not been definitively determined, even though several models were proposed to date. To elucidate the chromosome higher order structure, metaphase chromosomes isolated by a polyamine-percoll method from HeLa S3 cells synchronized with colcemid, were treated with various salt concentrations. Changes in chromosomal morphology were observed by SEM. Chromosomes basically maintained X-shaped structure in each salt concentration, but their surface structure changed from granular to fibrous structure along with increasing salt concentration. Chromatid segregation and chromosome decondensation were both considerably changed when chromosomes were treated with 0.4 M NaCl. Chromosome structural alteration with salt treatment suggested that specific proteins dissociated from chromosomes contribute to the higher-order chromosome structure. hCAP-G, a non-SMC subunit of condensin I, TopoII α and Ki-67 are identified as chromosomal proteins: hCAP-G and TopoII α are localized at chromosome axis and are required for mitotic chromosome assembly; Ki-67 is localized at nucleoli in interphase and periphery of chromosomes during mitotic phase. The localization of these proteins in chromosomes was further confirmed by an immunoelectron microscopy using gold labeled antibodies. However, chromosome morphological alteration was supposed to occur during or after immunogold staining in our initial studies. Optimization conditions for immunogold staining revealed that chromosomal morphology was well-preserved and chromosomes were more compact in PBS buffer containing $Mg^{2+}$. Localization analyses of Ki-67 using immunogold-labeling were consistent with immunofluorescent staining revealed that Ki-67 is localized at chromosomal periphery and centromeric region. To elucidatie higher-order chromosome structure localization and interaction analyses of chromosomal proteins would give further insight.

1288

**Targeting Heterochromatin Formation on the Dot Chromosome of D melanogaster**

S. C. R. Elgin,[1] A. Caudy,[2] W. Leung,[1] K. A. Haynes[1]; [1]Biology, Washington University, St. Louis, MO, [2]Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ

The small fourth chromosome of *Drosophila melanogaster* is unusual; while it has a normal gene density in its short distal arm (1.2 Mb), this domain has a ca. 10-fold higher density of repetitious sequences than the euchromatic arms, and is largely packaged as heterochromatin. Using a *P* element reporter containing an *hsp70*-driven copy of the *white* gene to map the region on the fourth chromosome around *Hcf*, we observe that when the reporter lies within ca. 10 kb of a *1360* repetitious element, it shows a variegating phenotype, indicative of heterochromatin packaging; however, when the reporter lies further away, it shows a red eye phenotype, indicative of euchromatic packaging (Sun et al., 2004, Mol. Cell. Biol. 24, 8210). In a direct test, a single copy of *1360* in a *P* element construct did not by itself lead to a variegating phenotype, but did make a critical contribution to silencing when the *P* element was inserted into a pericentric region, as shown by a loss of silencing on FLP-medicated excision of *1360*. Silencing via heterochromatin formation has been shown to be dependent on the RNAi system in Drosophila (Pal-Bhadra et al., 2004, Science 303, 669). Northerns done using the *1360* from our *P* construct to probe total RNA from embryos, Kc or S2 cells show dsRNA of ca. 22 bp; the amount of *1360* products in cells is impacted by *Dicer* knockdown. An inverted arrangement of adjacent *1360* fragments within an intron of *Caps* is predicted to form a stable hairpin, a possible source of siRNA's. We infer that effective silencing depends on the presence of an appropriate *cis*-acting RNAi target sequence and on the local density of repetitious sequences in the genome. [Supported by NIH grant GM 068388.}

1289

**Human Transcriptional Coactivator PC4, a Chromatin Associate Protein Induces Chromatin Compaction**

C. Das,[1] K. Hizume,[2] K. Batta,[1] K. Takeyasu,[2] T. K. Kundu[1]; [1]Molecular Biology and Genetics Unit, J N Centre for Advanced Scientific Research, Bangalore, India, [2]Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Human transcriptional coactivator PC4 is a highly abundant multifunctional protein, which plays diverse important roles in cellular processes including transcription, replication and repair. It is a unique activator of p53 function. Here we report that it is a bonafide component of chromatin with distinct chromatin organization ability. By direct cell imaging we show that PC4 is predominantly associated with the chromatin through all the stages of cell cycle and is broadly distributed on the mitotic chromosome arms in a punctate manner except the centromere. PC4 selectively interacts with core histones H3 and H2B (in vitro and in vivo) which is essential for PC4-mediated chromatin condensation as shown by micrococcal nuclease (MNase) accessibility assay, circular dichorism spectroscopy assay and atomic force microscopy. The AFM images show that PC4 compact the 100 kb reconstituted chromatin very distinctly as compared to the liker histone H1. Silencing of PC4 expression in HeLa cells results in chromatin decompaction as evidenced by the increased MNase accessibility. Global gene expression analysis reveals that knocking down of PC4 up-regulates several genes, suggesting its physiological role as a chromatin compacting protein. These results establish PC4 as a new member of chromatin associated protein (CAP) family, which plays an important role in chromatin organization.

1290

**Regulation of Genome Architecture in *Escherichia Coli*: The Expression of Topoisomerase-i is Essential for the Dps-dependent Nucleoid Compaction**

R. L. Ohniwa,[1] K. Morikawa,[2] J. Kim,[1] T. Ohta,[2] C. Wada,[1] A. Ishihama,[3] K. Takeyasu[1]; [1]Graduate School of Biostudies, Kyoto University, Kyoto, Japan, [2]Institute of Basic Medical Sciences, Tsukuba University, Tsukuba, Japan, [3]Nippon Institute of Biological Science, Tokyo, Japan

Atomic force microscopy (AFM) of *Escherichia coli*, *Staphylococcus aureus* and *Clostridium perfringens* nucleoids has revealed that the hierarchy of the bacterial genome organization is achieved via several steps of DNA folding using fundamental 40 and 80 nm fibers (Kim et al., 2004; Takeyasu et al., 2004). A nucleoid protein, Dps, plays a critical role to build up a tightly compacted nucleoid under specific environmental conditions in certain species. We report here the mechanisms of Dps/MrgA-dependent nucleotide compaction in bacteria. In *S. aureus*, an overexpression of exogenous MrgA (homologue of *E. coli* Dps) in the log phase led to a nucleotide compaction, whereas, in *E. coli*, such an overexpression of Dps did not induce nucleoid compaction. This was because Dps/MrgA-dependent nucleoid compaction required the expression of topoisomerase-I, and because the topoisomerase expression was repressed in the *E. coli* log phase, but not in the S. sureus log phase, due to the high-level expression of *E. coli* Fis that inhibited the topoisomerase-I gene expression. (Note that S. aureus does not possess the *fis* gene.) This molecular mechanism can be generally applicable to other types of nucleoid compaction; e.g., oxidative stress-induced nucleoid compaction. The expression of both endogenous *dps* and *mrgA* genes was up-regulated in the presence of 2mM $H_2O_2$. However, only the *S. aureus* nucleoid was compacted in this condition. When the *fis* gene was deleted and the topoisomerase-I expression was up-regulated in *E. coli*, such an oxdative stress successfully led to the nucleoid compaction. These results suggest that the regulation of DNA topology is critical for the Dps-induced nucleoid compaction.

1291

**Distinct Three Steps of Apoptotic Nuclear Condensation: Analysis of Cell-free Apoptosis by Time-lapse Imaging, Biochemical Technique and Electron Microscopy**

S. Tone,[1] K. Sugimoto,[2] K. Tanda,[1] H. Kanouchi,[1] Y. Minatogawa[1]; [1]Biochemistry, Kawasaki Medical School, Kurashiki, Japan, [2]Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka, Japan

Dynamic changes of nuclear chromatin are a one of most essential phenomena in apoptotic execution. In healthy cells, genomic DNA, ~2m in size, are functionally packaged into nuclei, several micrometers in diameter. At mitosis, chromatin is more condensed and transformed into chromosomes. In apoptosis, giant complex of DNA and nuclear proteins are faithfully and efficiently unpackaged and chromatin goes condensation. Therefore, it is tempting to speculate that cells would have an apoptosis-specific system by which cells efficiently induce chromatin condensation. We have been studying molecular mechanisms on apoptotic chromatin condensation using cell-free system, which has been developed (Lazebnik et al., '93, Samejima et al '98) and made us possible to reveal critical roles of each molecules in apoptotic execution. In this presentation, at first we will show the nuclear dynamic changes in cell-free apoptotic system and make a definition of each steps (Ring--Slits/Necklace--Final). Next, we will show the time-lapse imaging of DNA and centromeric protein during cell-free apoptosis. In our hands, total time of cell-free apoptosis was ~30-40 min. Duration time of step to Ring and to final condensation step was both estimated within 4 mins. Chromatin and centromeres behaved together. Interestingly, EM revealed that neither chromatin nor subnuclear structures were present inside the "ring". DNase activity was not essential for the ring formation, because apoptotic extracts devoid of DNases could make ring structure. But DNase(s) are necessary to proceed to next Step, Slits/Necklace. Finally, if ATP will be omitted in the reaction of cell-free system, nuclear condensation arrested before Step of Final. It suggested that ATP-binding proteins acted in the final step. We will discuss the cell-biological significance of these findings in nuclear condensation.

1292

**Genetic and Biochemical Analysis of Chicken Smc5**

A. K. Stephan, C. Morrison; Biochemistry and NCBS, NUIG, Galway, Ireland

The Structural Maintenance of Chromosomes (SMC) proteins are highly conserved V-shaped molecules with long coiled-coil arms, existing as heterodimers in eukaryotes in complex with several non-Smc subunits. Three essential SMC- containing complexes have been identified to date: cohesin (containing Smc1 and Smc3), condensin (containing Smc2 and Smc4), and an as-yet unnamed complex containing Smc5 and Smc6 proteins. Like cohesin and condensin, the Smc5-6 complex is essential for viability and seems to control chromatin structure. Studies on mutants of the Smc5-6 complex in fission yeast have demonstrated that it plays a role in repairing double-strand breaks (DSBs) by homologous recombination. It is also suggested that the Smc5-6 complex is involved in checkpoint maintenance, by coordinating the checkpoint arrest of the cells until the completion of DNA repair. Nevertheless, the essential function of this complex remains unclear. To generate a model system for defining the roles of the Smc5 protein in DSB repair and mitosis we are using the hyper-recombinogenic chicken DT40 cell line. Initial results of targeting the *Smc5* locus indicate that disruption of the *Smc5* gene is lethal. In order to develop a conditionally null cell line with *Smc5* expressed from a tetracycline repressible promoter, *Smc5* cDNA and 5'untranslated region (5'UTR) were cloned and sequenced. Sequencing analysis showed 63% identity between chicken and human at the aminoacid level. We have also generated anti-chicken Smc1 and Smc5 antibodies, which will be used for characterizing Smc5 and investigating any direct or indirect interactions between the Smc5 protein and the cohesin complex that may control chromosome behaviour.

1293

**Live Imaging of the Amphibian Lampbrush Chromosomes**

N. Novikova, C. Austin, B. Beenders, S. Patel, M. Bellini; Cell & Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL

While the amphibian oocyte provides a high spatial resolution to investigate nuclear structures, live imaging of its nuclear compartment requires the nucleus to be hand-isolated in mineral oil and directly mounted under a cover glass. In this *in vivo*-like condition, the nucleus maintains all its activities for more than 24h, and non-chromosomal organelles such as nucleoli, Cajal bodies, ICGs as well as a heterogeneous population of large RNP granules are readily observable by light microscopy. The chromosomes remain elusive however. When oocytes were microinjected with DNA fluorescent dyes such as DAPI or Picogreen prior to isolating nuclei, two different structures were labeled: nucleoli and several thin and long fibers. Surprisingly, these fibers changed shape overtime and in some rare instances resumed the aspect of chromosomes. We concluded that these fibers were stretched chromatin fibers and that the apparent absence of chromosomes in oil-isolated nuclei resulted from their extensive damage (stretching and numerous breakages) during sample preparations. We recently modified the preparation procedure to minimize the stress imposed on chromomes and we obtained the very first live images of the oocyte lampbrush chromosomes. As a result, it is now possible to investigate the dynamics of nuclear proteins involved in chromatin organization with an unprecedented resolution.

1294

**The Use of miRNA and siRNA Libraries to Identify Genes Involved in the Regulation of hTERT Transcription**

D. Ovcharenko, K. Kelnar, A. Cheng, D. Wang, L. P. Ford; R&D, Ambion, Austin, TX

Human telomerase is composed of a catalytic reverse transcriptase, hTERT and an RNA component, hTR. The telomerase reverse transcriptase adds the telomeric repeat sequences found on the terminus of each human chromosome using hTR as the template. While most normal cells lack telomerase activity, more than 90% of all cancer cells are telomerase positive. In addition, induction of telomerase activity is strongly associated with the cellular transformation process. Although transcription of the hTERT gene is known to be the critical event in creating active telomerase in cells, it is not well understood how hTERT transcription is regulated. Therefore, we performed a screen using siRNA and miRNA libraries to identify genes that up regulate transcription of hTERT in a telomerase negative normal diploid human fibroblast cell line using a real time PCR assay. Using this approach we identified genes and miRNAs that induced hTERT transcription. The genes identified in the siRNA portion of the screen can be grouped into gene classes associated with cancer and aging. The miRNAs that were identified in the screen are predicted to regulate the expression of some of the genes identified in the siRNA screening. This suggests that these miRNAs are causing the induction of hTERT by regulating those predicted targets. This is the first account of combining siRNA and miRNA library screens to identify gene pathways and functions.

1295

**An Analysis of the in vivo Mechanism of the Bacterial Condensin MukBEF**

R. B. Case,[1] N. R. Cozzarelli,[1] C. Bustamante[2]; [1]Molecular & Cell Biology, UC Berkeley, Berkeley, CA, [2]Physics, U.C. Berkeley, Berkeley, CA

MukBEF, the condensin in Escherichia coli, is a member of a protein family broadly conserved from bacteria to man that organizes and compacts DNA in a mechanism essential for faithful chromosome partitioning. While progress has been made through genetic analyses of mutants and through in vitro assays of purified components, critical details of the biological mechanism remain undiscovered. For example, the role of ATP in MukBEF-DNA association and compaction is unknown; and the presence of other protein interacting partners and the biological compact structures that are formed are both still undescribed. I will present my progress on a set of in vivo experiments that directly address each of these shortfalls. Specifically, I am making a collection of point mutations that are each predicted to block the MukBEF ATPase cycle in a different step (ATP binding, ATP hydrolysis, etc.). Each mutant or mutant protein will be analyzed with flow cytometry, single-cell fluorescence microscopy, mass spectrometry and AFM to reveal how each mutation affects the in vivo mechanism. Once completed, the results should provide sufficient insight for an initial model of the biological mechanism of MukBEF-dependent DNA compaction and organization.

1296

**The JIL-1 Histone H3S10 Kinase Regulates Dimethyl Histone H3K9 Modifications and Heterochromatic Spreading in *Drosophila***

K. M. Johansen, W. Zhang, X. Bao, H. Deng, S. Lerach, J. Girton, J. Johansen; Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA

In *Drosophila* euchromatic genes can be transcriptionally silenced as a result of their placement in or near heterochromatin, a phenomenon known as position effect variegation (PEV). Repression typically occurs in only a subset of cells and can be heritable leading to mosaic patterns of gene expression. PEV in *Drosophila* has served as a major paradigm for the identification and genetic analysis of evolutionarily conserved determinants of epigenetic regulation of chromatin structure. In this study we present evidence that the JIL-1 histone H3S10 chromosomal kinase functions in a novel pathway to establish or maintain euchromatic regions by antagonizing heterochromatization and gene silencing. In the absence of JIL-1 the overall levels of histone H3K9 dimethylation and HP1 are unchanged; however, these major markers for heterochromatin formation spread to ectopic locations with a pronounced increase on the male and the female X chromosomes. Genetic interaction assays demonstrated that JIL-1 functions antagonistically to Su(var)3-9 which is the major catalyst for dimethylation of the histone H3K9 residue. On the basis of these findings we propose a model where JIL-1 functions to mark euchromatic domains and counteract heterochromatization and gene silencing at ectopic locations by Su(var)3-9-mediated histone H3K9 dimethylation and HP1 recruitment. In support of this model we demonstrate that the lethality of a severely hypomorphic *JIL-1* heteroallelic combination could be almost completely rescued by a reduction in *Su(var)3-9* dosage that prevented ectopic dimethylation of histone H3K9. This indicates that the lethality of *JIL-1* null mutants may be largely due to repression of essential genes at ectopic sites as a consequence of the spreading of Su(var)3-9 activity. (Supported by NIH grant GM62916).

1297

**Distinct Roles for Cysteines in the Structure and Nuclear Transport of the Chromatin Protein Hmgb1**

G. Hoppe, K. E. Talcott, S. K. Bhattacharya, J. W. Crabb, J. Sears; Cole Eye Institute, Cleveland Clinic, Cleveland, OH

Reversible modification of sulfhydryls by glutathione represents an efficient mechanism for regulation of protein activity in response to oxidative stress. Removal of glutathione adducts is catalyzed by a small oxidoreductase glutaredoxin. We sought to identify nuclear proteins that undergo redox-dependent S-glutathionylation and enzymatic de-glutathionylation in the retinal pigment epithelium (RPE). Selective oxidation of sulfhydryls was induced in cultured RPE cell lines diamide. Nuclear proteins were isolated, treated with recombinant glutaredoxin, labeled with biotin-maleimide, purified, and identified by mass spectrometry. Cellular localization and mobility of EGFP-fusion proteins were monitored using live fluorescent microscopy and fluorescence recovery after photobleaching. High mobility group protein B1 (Hmgb1), a DNA-binding structural chromosomal protein and transcriptional co-activator was identified as a substrate of glutaredoxin. Hmgb1 contains 3 cysteines, Cys23, 45, and 106 and their irreversible alkylation reduces Hmgb1 DNA binding. After oxidative stress, Cys23 and Cys45 readily form intramolecular disulfide bridge, whereas Cys106 remains in the reduced form. EGFP-tagged wild type Hmgb1 and endogenous Hmgb1 co-localized in the nucleus. Replacement of Cys23 and/or 45 with serines did not affect the nuclear distribution of the mutant proteins, while C106S and triple cysteine mutations impaired nuclear localization of Hmgb1. Intracellular mobility of Hmgb1 was severely impaired by the mutation of Cys106, but not of Cys23 or 45. Hmgb1 and its homologue Hmgb2 are the only HMG-box proteins that contain cysteines that may be targets of reversible protein S-glutathionylation. Cys23 and 45 confer redox-sensitivity by inducing conformational and functional changes in response to oxidative stress, while Cys106 governs nucleocytoplasmic shuttling of Hmgb1.

## Nuclear Import and Export Signals (1298-1311)

1298

### Nuclear Import of the HIV-1 Integrase

A. Armon-Omer, A. Loyter; Biological Chemistry, Hebrew University of Jerusalem, Jerusalem, Israel

The HIV-1 integrase (IN), have been implicated in mediating nuclear import of the viral pre-integration-complex. Previously we have shown that a synthetic peptides bearing residues 161-173 of the HIV-1 IN was able to mediate active import into nuclei of permeabilized cells, as well as binding to importin α, and therefore was designated as the nuclear localization signal NLS(IN). Subsequently we have study the karyophilic properties of a recombinant IN proteins lacking the NLS(IN) domain. This truncated protein designated as IN-152, exhibited partial nuclear import when incubated with permeabilized cells or when microinjected into intact cultured cells. Receptor-mediated nuclear import is evident from experiments showing that translocation of the IN-152 was competitively inhibited by it self. The IN-152 interacted with importin α, but to a lesser extent than that of the full-length IN. Thus it appears that in addition to the NLS(IN) region, additional domains may be involved in active nuclear transport of the IN. In-vivo studies of IN interaction with importin α were performed using the srp1-31 yeast mutant strain, whose importin α is not functional at non-permissive temperature. Cells were transfected with an inducible expression vector bearing the coding sequence of a yEGFP-IN. In mutant strain incubated at the permissive temperature nuclei, was highly fluorescence, while in those incubated at the non-permissive temperature a large part of the fluorescence was located in the cytosol. These findings strongly support the view that nuclear import of the IN occurs via the importin α pathway.

1299

### The Cellular Localizations of SR-Related Proteins in *Schizosaccharomyces pombe* Fission Yeast Are Affected by SR-Protein Specific Kinase Dsk1

Z. Tang, S. Alaei, C. Wilson, C. Chiu, J. Oya, J. Shin, H. Buitron, S. Hardy; W.M. Keck Science Center, Claremont Colleges, Claremont, CA

Prp2, Srp1, and Srp2 are SR-related proteins in *Schizosaccharomyces pombe*. Prp2 protein is the orthologue of U2AF large subunit that genetically interacts with Dis1-suppressing kinase (Dsk1), the orthologues of human SR-specific protein kinase1 (SRPK1). All three proteins are specifically phosphorylated *in vitro* by Dsk1 kinase. Srp1 and Srp2 form a complex and their association is inhibited by Dsk1-mediated phosphorylation. To investigate the regulatory role of Dsk1 *in vivo*, we determined the cellular localizations of Prp2, Srp1, and Srp2 by ectopical expression of the corresponding GFP fusion proteins in wild-type and *dsk1*-deletion fission yeast strains. Consistent with its role in pre-mRNA splicing, GFP-Prp2 was localized in the nucleus of wild-type cells, but it was diffused throughout the *dsk1*-null mutant cells. A similar distribution pattern was also observed for GFP-Srp2. Opposite to Prp2 and Srp2, GFP-Srp1 was normally spread out in the cell, while it became retained in the nucleus of the cell with $dsk1^+$ deleted. The cellular localization of these proteins were also investigated in a strain with another SRPK gene $kic1^+$ deleted. Although the localization pattern of Srp1 was partially changed in the *kic1*-null strain, the distributions of Srp2 and Prp2 remained the same in the mutant as in the wild-type cells. Therefore, the cellular localizations of the SR-related proteins are specifically affected by Dsk1 kinase. Our studies provide the first genetic evidence for the important role of Dsk1 in modulating the cellular localizations of the SR-related proteins, revealing an *in vivo* function of SRPKs. Together with the effect of the phosphorylation on the Srp complex, the results suggest a potential mechanism to govern the activities of the SR-related proteins by Dsk1 through regulating their interactions and localizations in fission yeast cells.

1300

### Pushing the Envelope on Nuclear Import of Parvovirus

S. Cohen, A. Behzad, J. Carroll, N. Pante; Department of Zoology, University of British Columbia, Vancouver, BC, Canada

Parvoviruses are small DNA viruses that replicate in the nucleus of their host cells. After entering the cell by receptor-mediate endocytosis, the virus is released into the cytoplasm and enters the nucleus. Cell-entry of parvoviruses has been studied in some detail. However very little is known about trafficking of parvoviruses from late endosomes on. In particular, the mechanisms underlying parvovirus' nuclear import remain largely undefined. Nevertheless, it has been assumed that parvovirus enters the nucleus through the nuclear pore complex (NPC). In order to study this problem we microinjected the parvovirus minute virus of mice (MVM) into the cytoplasm of Xenopus oocytes and used electron microscopy (EM) to visualize its nuclear import. In contrast to oocytes microinjected with other import substrates that have shown the substrate crossing the NPC, MVM was not seen in transit through NPCs. Instead, we found that MVM caused damage to the nuclear envelope (NE) in a time- and concentration-dependent manner. Our findings suggest that MVM is imported into the nucleus using a unique mechanism that is independent of the NPC, and involves disruption of the NE. To demonstrate that this mechanism is used by MVM during infection, we then infected mouse fibroblast cells with MVM and examined the cells by fluorescent microscopy after performing double immuno-labeling with anti-lamin A/C and anti-MVM antibodies. Consistent with our results on Xenopus oocytes we found abnormal gaps in the lamin A/C immuno-staining. To further characterize these NE perturbations at the ultrastuctural level, MVM infected cells were also analyzed by EM. We found that MVM infection was associated with dramatic alterations in the nuclear shape and NE morphology. Our findings are consistent with a mechanism of nuclear entry of MVM that involves disruption of the NE and import through the resulting breaks.

1301

### Apoptin Nuclear Accumulation is Modulated by a CRM1-Recognised Nuclear Export Signal that is Active in Normal but not Tumor Cells

I. K. H. Poon, C. Oro, M. M. Dias, D. A. Jans; Dept. of Biochem. & Mol. Biol., Monash University, Clayton, Australia

Cancer is a growing problem for human health world-wide, with a dearth of efficient and above all specific anti-cancer treatments. Viral protein 3 (VP3/apoptin) from Chicken Anaemia Virus (CAV), represents a potential, novel anti-cancer tool, as it appears to possess tumor-specific p53-independent, Bcl-2-enhanced pro-apoptotic activity (1). Intriguingly, the anti-tumour cell activity of VP3 is integrally linked to its ability to localise in the nucleus of transformed cells, but not in primary or non-transformed cells (1). We have characterised the signals responsible for VP3's novel nucleocytoplasmic trafficking properties in detail for the first time by performing transfection studies and quantitative confocal microscopic analysis in two isogenic tumor/non-tumor cell pairs. Additional to identifying the tumor cell-specific nuclear targeting signal (tNTS), comprising two stretches of basic amino acids in the VP3 C-terminus which are more efficient in tumor than normal cells, we define the CRM1-recognised

nuclear export sequence (NES; amino acids 97-105) within the VP3 tNTS which, intriguingly, is functional in normal but not tumor cells. The basis for lack of functionality in tumor cells appears to be through the threonine 108 phosphorylation site adjacent to NES which inhibits its action. We also characterize an N-terminal leucine-rich sequence within VP3 (amino acids 33-46) that contributes to VP3 nuclear accumulation by functioning as a nuclear retention sequence, conferring association with promyelocytic leukemia (PML) nuclear bodies. This unique combination of signals, and in particular the phosphorylation regulated NES, is the basis of VP3's tumor cell-specific nuclear targeting abilities, and may have important possible application in targeting drugs efficiently to the nucleus of tumor cells. **(1) Oro C, Jans DA.** *Curr. Drug Targ*ets **5**, 179, 2004 **(2) Poon IKH *et al.*** *J. Virol*. **79**, 1339, 2005

1302

**Identification of a Novel Nuclear Export Sequence of OREBP/TonEBP/NFAT5 that Controls Cytoplasmic Localization**

B. Ko,[1] E. H. Tong,[1] S. S. Chung[2]; [1]Department of Chemistry, The University of Hong Kong, Hong Kong, Hong Kong Special Administrative Region of China, [2]Department of Physiology, The University of Hong Kong, Hong Kong, Hong Kong Special Administrative Region of China

The Osmotic-Response Element-Binding Protein (OREBP), also known as the Tonicity Enhancer-Binding Protein (TonEBP) or NFAT5, regulates the hypertonicity- induced expression of a battery of genes crucial for the adaptation of mammalian cells to extracellular hypertonic stress. Recently, it has been suggested that OREBP may play a role in metastasis. The nucleocytoplasmic trafficking of OREBP plays an important role in regulating its function. Here we show that, by immunocytochemistry and GFP fusion, the transactivation domain of OREBP is not necessary for the nucleocytoplasmic trafficking. Nuclear export of OREBP can be blocked by leptomycin B, suggesting that it is a Crm1-dependent process. However, two leucine-rich motifs located in the N-terminal of OREBP do not function as nuclear export signals (NES). In contrast, a protein domain N-terminal to the DNA-binding domain functions as NES and directs the localization to the cytoplasm. Recombinant OREBP devoid of the NES constitutively resides in the nucleus despite of extracellular hypotonicity.

1303

**Cse1: Exporting Importin-α**

A. G. Cook,[1] G. Schlenstedt,[2] E. Conti[1]; [1]Structural and Computational Biology, EMBL, Heidelberg, Germany, [2]Medizinische Biochemie und Molekularbiologie, Univeritaet des Saarlandes, Homburg, Germany

During interphase thousands of molecules of RNA and protein must cross the nuclear membrane to move from the site of synthesis to the site of function. Cse1 is a nuclear exportin of the karyopherin-β family that is required for RanGTP-dependent recycling of importin-α to the cytoplasm. Importin-α acts as an adaptor for importin-β by recognizing proteins with a classical nuclear localization signal (NLS). The structure of the cytoplasmic, cargo-free structure of Cse1 has been solved to 3.1 Å revealing a HEAT repeat protein consisting of an N-terminal and a C-terminal arch (Cook *et al* 2005 Mol. Cell 18:355). Comparison with the structure of the cargo-bound, nuclear form of Cse1 reveals a large conformational change. The cytoplasmic, cargo-free Cse1 forms a ring-like structure created by an intramolecular interaction between the N-terminal 3 HEAT repeats and part of the C-terminal arch. This interaction is disrupted in the nuclear form when RanGTP and importin-α are bound. The conformational changes are localized to a hinge region between the N- and C-terminal halves of Cse1. Isothermal calorimetry (ITC) analysis indicates that both RanGTP and importin-α are able to independently interact with Cse1. They form relatively low affinity complexes compared with the heterotrimer complex that has a binding constant in the low nanomolar range. Mutations in the intramolecular interface uncouple importin-α binding from RanGTP whereas mutations to a conserved insertion in the hinge region prevent complex formation. These data point to a mechanism of binding that involves initial recruitment of RanGTP and importin-α through low affinity interactions followed by a larger structural rearrangement to form a tight complex.

1304

**Nucleocytoplasmic Shuttling of Hsc70s Is Inhibited in Stressed Cells**

M. Kodiha, R. Zhang, X. Quan, V. Illanganathan, J. Yu, N. Matusiewicz, U. Stochaj; Physiology, McGill University, Montreal, PQ, Canada

Heat shock proteins of the hsp/hsc70 family are essential chaperones, implicated in the stress response, aging, and a growing number of human diseases. At the molecular level, hsc70s are required for the proper folding and intracellular targeting of polypeptides as well as the regulation of apoptosis. Cytoplasmic members of the hsp/hsc70 family shuttle between nuclei and cytoplasm, and they are found in both compartments of unstressed cells. However, hsc70s concentrate in nuclei when cells are exposed to stress. Our objective is to define the molecular mechanisms that underlie the stress-induced nuclear accumulation of hsc70s. We now demonstrate that actin filament-destabilizing drugs concentrate hsc70s in nuclei of cells that are unstressed or recovering from heat exposure. Using human/mouse heterokaryons we show that heat shock inhibits hsc70 shuttling and sequesters the chaperone in nuclei. The inhibition of shuttling in response to stress is transient only, and transport is re-established when cells recover from heat. We have defined one of the mechanisms that prevent hsc70s to move in and out of the nucleus upon stress. As such, hsc70 shuttling is controlled by nuclear retention of the chaperone. This retention depends on two distinct processes, ATP-sensitive binding of hsc70s to chaperone substrates and an ATP-insensitive association with nucleoli. Our studies identified the nucleolar protein fibrillarin and ribosomal protein rpS6 as interacting components that show an increased association with hsc70s in the nucleus of stressed cells. This supports the idea that hsp/hsc70s play a pivotal role in restoring nucleolar functions after heat shock. Taken together, our data suggest that stress abolishes the export of hsc70s to the cytoplasm, thereby limiting their function to the nuclear compartment. We propose that during recovery from stress hsc70 is released from nuclear and nucleolar anchors, a prerequisite to resume shuttling.

1305

**CRM1-Dependent Nuclear Export of *IFN-α1* mRNA Is Mediated through a Structured Target Sequence That Is Conserved in *IFN-α2* and *IFN-α13* mRNAs**

T. Kimura, I. Hashimoto, J. Fujisawa; Microbiology, Kansai Medical University, Moriguchi, Japan

We recently reported the chromosome region maintenance 1 (CRM1)-dependent, but not adenylate uridylate-rich element (ARE)-mediated, nuclear export of the intronless human *interferon-α1 (IFN-α1)* mRNA. To investigate whether there are cis-acting sequences within the *IFN-α1* gene that mediate this CRM1-dependent mRNA export, we conducted deletion mutagenesis of the gene. Based on an in vivo export assay and subsequent

computer analyses of the folding potentials of export-competent fragments of *IFN-α1* mRNA, we found that a 68-nucleotide (nt) structured element allowed the mRNA export. This novel cellular sequence was located to nt 353-420 within the coding region of the *IFN-α1* gene, forming a putative RNA secondary structure that facilitates the mRNA export. This 68-nt element RNA was colocalized in the nucleus with CRM1, whose export was leptomycin B-sensitive. Upon removal of the element, *IFN-α1* mRNA failed to colocalize with CRM1, resulting in the nuclear retention of the RNA. In addition to the *IFN-α1* mRNA, the structured element is also conserved in the human *IFN-α2* and *-α13* mRNAs, which together represent the three dominant species of *IFN-α* mRNA expressed upon viral infection. Since coexpressed human immunodeficiency virus type 1 Rev blocked the expressions of both the *IFN-α2* and *-α13* genes in the same dose-dependent manner as for the inhibition of *IFN-α1* mRNA export, we suggest that the newly identified structured RNA element may be involved in the posttranscriptional regulation of these *IFN-α* gene expressions in a CRM1-dependent manner.

1306

**Osmotic Stress Signaling to the Nuclear Transport Machinery**

J. B. Kelley, B. M. Paschal; Center for Cell Signaling, University of Virginia, Charlottesville, VA

A high nuclear:cytoplasmic (N:C) gradient of the Ras-related GTPase Ran is used to regulate multiple nuclear import and export pathways in eukaryotic cells. Here we report that osmotic stress induced by 0.4 M sorbitol results in a rapid (5-10 min) breakdown of the Ran gradient in HeLa cells. Nuclear import rate measured by microinjection of a fluorescent reporter protein was reduced by 45%. Concomitant with breakdown of the Ran gradient was a dramatic change in the N:C distributions of NTF2 and Mog1, two of the proteins that regulate the Ran gradient in cells. The Ran import pathway may, therefore, be a target of sorbitol-induced stress signaling. Strikingly, 20 min following addition of sorbitol there was a partial recovery of the Ran gradient that correlated with a partial recovery of NTF2 and Mog1 N:C distributions, and nuclear import rate. Recovery was followed by a second drop in the Ran gradient, NTF2 and Mog1 N:C distributions, and nuclear import rate. A second recovery phase was observed between 30 and 60 minutes, suggesting there is a biphasic recovery. Kinase activity profiles of sorbitol-treated cells (35 kinases examined) lead us to study two pathways in Ran gradient regulation, p38 MAP kinase and PKC zeta. Cells pretreated with the p38 inhibitor SB203580 do not display the Ran gradient recovery normally observed after 20 min of sorbitol treatment, however, these cells still displayed recovery at 60 min. A myristoylated pseudosubstrate that inhibits PKC zeta, and a dominant negative form of PKC zeta, each disrupted the Ran gradient even in the absence of sorbitol-induced stress signaling. Thus, constitutive PKC zeta activity is required for the maintenance of the Ran gradient, while p38 mediates the initial recovery of the Ran gradient in response to osmotic stress.

1307

**Nuclear Localization Signal: A Route to the Nucleolus**

H. Htun, Y. K. Lee, Y. X. Luong, T. Lee; Ob-Gyn and Molecular & Medical Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, CA

Nuclear localization signal (NLS) plays an important role in targeting proteins to the nucleus of eukaryotic cells. Once within the nucleus, the nuclear protein can exist throughout the nucleoplasm as well as in various membrane-less nuclear compartments, the most prominent being the nucleolus. In targeting a fluorescent RNA-binding protein to the nucleus, we have found NLS derived from SV40 large T-antigen to not only cause nuclear uptake but also to target our RNA-binding protein to the nucleolus. As this association with the nucleolus was unanticipated, we examined the role of the RNA-binding as well as type of NLS in nucleolar targeting. Site directed mutagenesis of the RNA-binding domain revealed a minor role for the RNA-binding domain in nucleolar association. However, examination of other NLSs showed that NLSs have differing potential to promote association with the nucleolus. In particular, the SV40 large T-antigen NLS has a greater affinity for the nucleolus than NLS derived from N-myc protein; and both NLSs have a higher affinity than NLS derived from c-myc protein. Positive correlation exists between a NLS' affinity for the nucleolus and its isoelectric point, with a greater propensity for nucleolar association observed for a more basic NLS; however, the extent of nucleolar association depends not only on the isoelectric point but also on the NLS sequence. Actinomycin D treatment disperses the chimeric NLS-containing protein from the nucleolus, indicating a requirement for RNA polymerase I transcription to maintain NLS-mediated nucleolar association. Lastly, we initially found fusions to c-myc derived NLSs to be excluded from the nucleolus; however, upon introduction of additional copies of the c-myc derived NLS, the fusion protein accumulated in the nucleolus. Thus, we propose that all basic NLSs have an inherent propensity to associate with the nucleolus.

1308

**Cohort Relationships Among Members of the Arabidopsis thaliana Hsp70 Molecular Chaperone Machine**

J. A. Miernyk,[1,2] Y. Suo,[2] M. L. Johnston[1]; [1]Plant Genetics Research Unit, USDA, ARS, Columbia, MO, [2]Biochemistry, University of Missouri, Columbia, MO

The Arabidopsis thaliana genome includes 17 genes that encode Hsp70 proteins and 94 genes that encode J-domain molecular chaperone proteins. The only know function of the J-domain is to direct docking between these two members of the Hsp70 molecular chaperone machine. Presumably, relationships among these proteins are specific rather than promiscuous. We have begun the process of defining cohort relationships. It has been experimentally determined that both members of Family 4 of the J-domain proteins, atDjC6 and atDjC37 are nuclear-localized. Based upon in silico analyses, AtHsp70-1, -2, -3, -4, -5, -14, and -15 are potentially nuclear or nucleocytoplasmically-localized. We have used bacterial 2-hybrid analysis to quantify interactions between these Hsp70 and J-domain proteins. Results from these analyses suggest specific interaction between atDjC6 and AtHsp70-1, and atDjC37 and AtHsp70-4 or AtHsp70-1. AtHsp70-6, a mitochondrial chaperone, was used as a control. Interactions with AtHsp70-1 were confirmed by co-precipitation of the recombinant proteins in vitro. We will further confirm these results using the GFP-trap assay, and extend them by using FRET for analysis of in planta interactions among fluorescent protein chimera.

1309

**Analysis of Nuclear Import of Adenoviral Core Protein VII Suggests Redundant Pathways for Import of the Viral Genome**

A. Cassany,[1] H. Wodrich,[2] M. D'Angelo,[1] T. Guan,[1] L. Gerace[1]; [1]Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA, [2]Adenovirus: Vectorology, Trafficking and Receptors, CNRS UMR 5535, Institute of Molecular Genetics, Montpellier, France

Adenovirus is non-enveloped DNA virus with an ~36kb linear double-stranded genome. During virus infection, adenovirus DNA is thought to enter the nucleus in a complex with its core proteins. The nuclear import of adenovirus DNA can be inhibited in vitro by an excess of basic amino acid-rich protein nuclear localization signals (NLSs), suggesting that NLS(s) of the adenovirus core proteins drive the DNA import. Protein VII, which is the most abundant adenovirus DNA-binding protein (~1000 copies/virion), also is the most tightly bound core protein. We are testing the hypothesis that protein VII is an adaptor that mediates the nuclear import of the adenovirus genome. We found that protein VII contains potent NLS activity by transfection and by microinjection of cultured cells with protein VII fusion constructs. We identified three NLS containing regions in protein VII by deletion mapping, and determined the NLS sequences by point mutagenesis. The recombinant protein VII and its NLS-containing domains strongly and specifically bind to several nuclear import receptors including importin a, importin b, and importin 7. We demonstrated that binding of protein VII to this diverse range of receptors is functionally significant using in vitro nuclear import assays in permeabilized cells reconstituted with recombinant receptors. Considered together, these data suggest that protein VII can act as an adaptor to access several different receptor pathways for nuclear import of the viral genome. This suggests that adenovirus uses highly redundant mechanisms to promote efficient nuclear import of its DNA.

1310

### Identification and Characterization of a Putative Nuclear Export Signal in the Tbx5 Transcription Factor

A. Kulisz,[1,2] H. Simon[1,2]; [1]Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL, [2]Developmental Biology, Children's Memorial Research Center, Chicago, IL

In vertebrates, the transcription factor, Tbx5, is important in controlling cardiogenesis and forelimb induction and identity. However, it is not understood how Tbx5 regulates these events in the cell, nor how Tbx5 is regulated. We recently have identified a novel PDZ-LIM protein family member, Lmp4 that specifically binds Tbx5 but not related Tbx proteins. When expressed individually in COS7 cells, Tbx5 is nuclear and Lmp4 localizes with actin filaments. However, when co-expressed in COS7 cells, Lmp4 binds and targets Tbx5 to the actin filaments. Although two nuclear localization signals (NLS) have been identified, it is not known by what mechanism Tbx5 leaves the nucleus. We have identified a leucine-rich nuclear export signal (NES) in Tbx5, which is found in most of the substrates of the CRM1 export protein. Interestingly, within the Tbx2/3/4/5 subfamily Tbx5 and Tbx4 are unique to contain this putative NES. To determine if this NES is functional, mutagenesis was utilized to change critical amino acid(s) within this sequence. The NES-mutant Tbx5 constructs were expressed as EGFP-fusions with and without Lmp4 co-expression and localization was monitored by confocal microscopy and FRAP. All Tbx5 NES-mutants were retained in the nucleus even in the presence of Lmp4. The subcellular localization and interaction with Lmp4 were further analyzed via co-immunopreciptation, transcriptional activity assayed using luciferase reporter constructs, also in the presence of Leptomycin B, which is known to interfere with the CRM1 pathway. Taken together these data suggest that Tbx5 possesses a functional NES, which allows for an alteration in subcellular localization in the presence of Lmp4. This is the first demonstration of a NES in a Tbx transcription factor and offers new insights into a potential mechanism for regulated cytoplasmic localization of the Tbx5 transcription factor.

1311

### Identification of Nucleoporin Binding Sites for Importin in situ at the Nuclear Pore Complex

S. S. Patel, M. Rexach; Biological Sciences, Stanford University, Stanford, CA

Nucleocytoplasmic transport is mediated by karyopherins and proceeds through nuclear pore complexes (NPC) embedded in the nuclear envelope. The first step in the translocation process is the docking of karyopherins to the cytoplasmic face of the NPC. Here we have characterized the docking step for the Kap95-mediated import pathway by reconstituting the binding of purified Kap95-Kap60 heterodimers to intact isolated yeast nuclei at 4°C. We find that on average, each NPC can dock up to 400 molecules of Kap95-Kap60 with an affinity ($K_D$) of 260 nM. Using a highly specific photocrosslinking approach, we found that FG nucleoporins (FG nups) located at the cytoplasmic face (Nup159, Nup42) and at the center of the NPC (Nup116, Nup100, nNup145, Nup57, Nup49, Nsp1) provide the primary docking sites for Kap95 *in situ* at NPCs. In contrast, the FG nups Nup53 and Nup59, and many other abundant non-FG nups and POMs in the center of NPC, did not crosslink to Kap95. Surprisingly, we also did not detect binding of Kap95 to any of the three nuclear basket nups (Nup1, Nup2, Nup60) *in situ*. We conclude that FG nups line the transport conduit of the NPC, and hypothesize that a thermally-operated gating mechanism prevents access of Kap95-Kap60 import complexes to the nuclear basket.

## Organogenesis (1312-1320)

1312

### Functional Screening for the Zygotic Determinants of Germ Layer Specification

N. Sasai, Y. Sasai; Organogenesis and Neurogenesis, Center for Developmental Biology, Kobe, Japan

The animal cap explant that is prepared from the blastula amphibian embryo is pluripotent and has the competence to differentiate into mesodermal tissues in responce to the mesoderm inducers such as FGF or activin. In contrast, the animal cap explant of the late-gastrula stage no more responds to these factors presumably because inhibitory signals are present at this stage. However, the molecular mechanisms underlying the loss of mesodermal competence have been largely unknown. This time, we performed a functional screen using early Xenopus embryos in order to search for zygotic genes which are involved in the ectodermal specification. We prepared a plasmid cDNA library from the animal cap of the late-gastrula stage, and screened 20,000 clones by injecting pools of mRNAs to select the clones that suppressed the expression of the early mesoderm marker Xbra (Xenopus Brachyury). By this screen, we identified seven novel zygotic genes including transcription factors and signaling modulators. Thus, many distinct signals as well as the already-known factors are involved in the ectodermal determination, in addition to maternal factors such as Ectodermin.

1313

### Disabled-2 is Essential for Angiogenesis *in vivo*

S. Cheong, J. Han; Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, Republic of Korea

The molecular mechanisms underlying the formation of embryonic blood vessels are poorly understood. We show here that Disabled-2 (Dab2), a cytosolic adaptor, has a pivotal role for the blood vessel formation in *Xenopus* and regulates the induction of VEGF via TGF-beta signaling pathway. Using the EST data base, we isolated cDNA for *Xenopus* Disabled-2 (XDab2) and examined its potential role in *Xenopus* embryogenesis. It is intriguingly expressed in blood vessels including pronephric sinus, vascular vitelline network, common cardinal vein, anterior cardinal vein, posterior cardinal vein, and intersomitic vein. Loss of function experiments using the morpholino oligonucleotides (MO) show that the formation of intersomitic veins which arise from angiogenesis is disrupted. Moreover, using the dominant negative TGF-beta receptor, we find that TGF-beta signaling is required for embryonic angiogenesis. In animal cap assay, we also show that TGF-beta signaling induces VEGF expression and its induction is decreased by Dab2 MO. However, other target genes of TGF-beta signaling pathway is unchanged by Dab2 MO. Furthermore, Dab2 knock-down embryos are rescued by VEGF. Together, these results suggest that XDab2 is essential for embryonic angiogenesis by regulating VEGF induction through TGF-beta signaling pathway.

1314

**SRG3, A Core Component of Mouse SWI/SNF Complex, Is Essential for the First Angiogenesis in Mouse Yolk Sac**

D. Han, J. Oh, S. Jeon, R. H. Seong; Department of Biological Sciences, Institute of Molecular Biology and Genetics, and Research Center for Functional Cellulomics, Seoul, Republic of Korea

We found that SRG3, a core component of mouse SWI/SNF chromatin remodeling complex, is expressed specifically in the yolk sac visceral endoderm in the early mouse embryo. We have previously shown that mutant mice deficient of the SRG3 expression are peri-implantation lethal. To investigate the role of Srg3 in the post-implantation stage, we set up the transgenic rescue experiment using transgenic mice(Tg+) expressing the SRG3 by chicken β-actin promoter. The rescued embryos(Srg3-/-Tg+) overcame peri-implantation lethality, but degraded after E10.5. Especially, fetus development was delayed and defects in yolk sac blood vessel were observed. In the visceral endoderm of the embryo yolk sacs, the SRG3 expression was virtually absent, and there were the marked reduction of angiogenesis-related genes. These results reveal that SRG3 is essential for the regulation of the yolk sac angiogenesis-related genes during the early mouse embryonic vessel development.

1315

**Role of p53 in Creating and Maintaining Luminal Space During Embryonic Submandibular Salivary Gland Morphogenesis**

T. Jaskoll, D. Witcher, G. Abichaker, M. Melnick; Lab Developmental Genetics, University of Southern California, Los Angeles, CA

Apoptosis has been shown to mediate the formation of lumina during embryonic submandibular salivary gland (SMG) development, with epithelial branches and terminal buds being hollowed out to form the ductal system and the presumptive acini. Centrally-localized epithelial cells undergo apoptosis whereas boundary cells with basement membrane contact survive. With the initiation of apoptosis from the center to the periphery, p53 protein is seen in centrally-localized cells targeted for death. Thus we postulate that p53 signaling is important for the creation and maintenance of luminal spaces during embryonic SMG morphogenesis. To begin to address this hypothesis, E15 mouse SMGs were cultured for up to 12 days (E15+12) in the presence or absence of pifithrin-α (PFTα), an inhibitor of p53 signaling. PFTα-treated E15+6 explants lack polarized epithelial cells and are characterized by lumina partially filled with cells and/or cellular debris. By day 12 of culture (E15+12), p53 inhibition results in the almost complete absence of luminal spaces. Since p53 signaling has been shown to induce cell cycle arrest, we then determined the affect of p53 inhibition on embryonic SMG epithelial cell proliferation. A significant 13% increase in cell proliferation is seen in PFTα-treated E15+3 explants. However, continued PFTα treatment *in vitro* results in a significant 32% decrease in cell proliferation in E15+8 explants and its virtual absence in E15+12 explants. Our results indicate that p53 signaling is not essential for creating lumina, but is necessary for apoptotic cell clearance and maintenance of a hollow lumen. The significant decrease in cell proliferation in PFTα-treated E15+8 and E15+12 SMGs indicates that increased epithelial cell proliferation does not account for the reducted size or absence of lumina. NIDCR (NIH) Grant number RO1 DE014535.

1316

**Heparan Sulfate Modulates FGF10 Function During Submandibular Gland Branching Morphogenesis**

V. N. Patel,[1] K. M. Likar,[1] M. Elkin,[2] I. Vlodasky,[2] M. P. Hoffman[1]; [1]Matrix and Morphogenesis Unit, CDBRB, NIDCR, National Institute of Health, Bethesda, MD, [2]Hadassah-Hebrew University Medical Centre, Jerusalem, Israel

Fibroblast growth factors (FGFs) are a family of heparin-binding growth factors that regulate branching morphogenesis of salivary glands. FGF signaling plays a key role in branching morphogenesis by regulating the gene expression and the activity of their receptors and extracellular matrix proteins. The affinity of FGF binding to FGF receptors is increased by heparan sulfate. In vivo the biological activity of heparan sulfate is regulated by heparanase, an endoglycosidase that degrades heparan sulfate. Chemical inhibitors of heparanase and function blocking anti-heparanase antibodies inhibit salivary gland branching suggesting heparan sulfate cleavage is required for FGF function during branching morphogenesis. The inhibitory effect of anti-heparanase antibodies was rescued by FGF10 but not by FGF7 suggesting heparan sulfate cleavage is required to release endogenous FGF10. Addition of exogenous FGF10 to epithelial rudiments induces cell proliferation at the tips of the ducts resulting in branching and duct elongation. This FGF10 mediated morphology is modified by exogenous heparin fragments containing 2-O and 6-O-sulfation, suggesting heparan sulfate increases the receptor binding of FGF10, and increases proliferation. Real time PCR shows that the genes for heparan sulfate 6-O sulfotransferases are expressed during early stages of SMG development with a profile similar to that of FGF10 expression. In situ analysis shows mesenchymal localization of 6-O-sulfotransferases adjacent to areas of proliferating epithelium. Collectively, our data suggest that defined sulfation patterns and cleavage of heparan sulfate, both regulate FGF10 function during SMG branching morphogenesis.

1317

**FoxF1 and Hex Expression Patterns during Gallbladder Organogenesis in the Zebrafish Embryo**

K. Young, E. Elzey, K. Halmon, L. Hammonds-Odie; Biology Department, Spelman College, Atlanta, GA

The genetic programs required for mesodermal interactions with endodermal cells in development, particularly as they relate to the formation of organs of the digestive system, have been the subjects of intensive study in recent years. During embryogenesis, endoderm-derived organ formation is accomplished through regional signaling pathways that must specify the gut tube to form the thyroid, lungs, liver, pancreas and gallbladder of vertebrates. Several transcription factors have been implicated in murine foregut development, particularly in the development of the liver and

pancreas. However, frequently in studies of biliary system or pancreatic development, the gallbladder is completely ignored, despite the proximity. The goal of this study is to document the expression of the zebrafish homologs of transcription factors for which a role in murine gallbladder development has been demonstrated. More specifically, we have examined the expression patterns of the transcription factors FoxF1 and Hex in zebrafish embryos. Determination of the expression patterns of these transcription factors temporally and spatially will not only help define the molecular control pathways that are involved in gallbladder organogenesis, but will also provide invaluable markers for early gallbladder development to be used in further studies.

1318

**Direct Temporal and Spatial Visualization of Vascular Endothelium and Its Support of Fetal Murine Liver Development**

M. L. Edwards, M. J. Ferkowicz, S. A. Johnson, W. C. Shelley, M. C. Yoder; Herman B Wells Center For Pediatric Research, IU School of Medicine, Indianapolis, IN

The role of the vascular endothelium for hepatoblast proliferation and differentiation in the fetal liver remains unclear. Use of fetal liver kinase-1 (Flk1) null mice has demonstrated a requirement for Flk1 expressing angioblasts in hepatic organogenesis, prior to vascularization of the liver. The temporal and spatial emergence of the endothelium in the developing fetal liver has not been previously described. We tested the hypothesis that the hepatoblasts emerge from the septum transversum and develop independent of a capillary blood supply. Nine to 13.5 days post coitus (dpc) mouse embryos were fixed in acetone and placed in a protein blocking solution, then exposed to fluorescent-labeled antibodies for a variety of cell lineage specific antigens. The tissues were imaged using a Biorad single photon confocal microscope. Between 9 and 9.5 dpc, a vascular plexus encompassing several parallel Flk1 + vessels arising from the vitelline vein and overlying the lateral ventral liver was visualized. The branches of the vitelline plexus appeared to penetrate the superficial hepatic buds. Visualization of the deep tissue of the liver revealed Flk+, CD31+, and VE cadherin+ hemangioblasts yet there were few vascular structures present, even at 12.5 - 13.5 dpc. The developing fetal liver appeared to be supplied by at least two distinct vascular systems. The deep system potentially arises from the left umbilical vein and superficial vessels penetrate from the vitelline plexus. These data support the hypothesis that the vascularization of the liver has at least two independent origins. Furthermore, paucity of vascular structures in the deep hepatic tissue supports the hypothesis that Flk+ hemangioblasts must be present for hepatic development but that a developed system of deep vessels appears subsequent to organization of the hepatic parenchyma.

1319

**Wnt Signals in Pronephric Kidney Tubule Development**

J. P. Lyons,[1] H. Ji,[1] X. Zhou,[2] P. D. Vize,[2] E. A. Jones,[3] G. Weidinger,[4] R. T. Moon,[4] T. DeRoo,[5] K. Vleminckx,[6] P. D. McCrea[1]; [1]Biochemistry and Molecular Biology, UT MD Anderson Cancer Center, Houston, TX, [2]Biological Sciences, University of Alberta, Calgary, AB, Canada, [3]Biological Sciences, University of Warwick, Coventry, United Kingdom, [4]Pharmacology and Center for Developmental Biology, HHMI, University of Washington School of Medicine, Seattle, WA, [5]Molecular Biomedical Research - VIB, University of Ghent, Ghent, Belgium, [6]Molecular Biomedical Reserch - VIB, University of Ghent, Ghent, Belgium

One of the basic and elegant morphologic forms that arises repeatedly during development is the tubule. In addressing the genesis of tubes in differing tissue contexts, studies from independent laboratories have supported the involvement of a number of signaling pathways. For example, in the kidney, published work from the laboratory of A. McMahon (Harvard U.), and later A. Brandli (ETH, Zurich), has indicated that the Wnt-4 ligand is required for vertebrate kidney tubule formation. Because Wnt-4 has the potential to activate both canonical (β-catenin-mediated) or non-canonical Wnt-signaling pathways upon binding Frizzled receptor, the question has remained which branch of the Wnt pathway is indispensable, or perhaps if both branches make significant inputs during tubule generation. We have begun by examining β-catenin-mediated (canonical Wnt) contributions, employing early *Xenopus laevis* embryos as our experimental system. By expressing dominant-suppressive or dominant-active β-catenin constructs that are dexamethasone-inducible (temporal control), and directing micro-injections into the prospective nephric field (spatial control), we have accumulated evidence that canonical Wnt signals are required. In evaluating molecular marker gene expression in conjunction with other assays, β-catenin-mediated signals appears to have a greater impact upon tubule differentiation than upon cell death or proliferation pathways. Continuing work will include study of non-canonical Wnt pathway contributions, which are known in other contexts to be required for the polarization and cytoskeletal activity of cells engaged in developmentally-directed cell movements. While morphogenesis is at once fascinating and complex, we expect that our work upon Wnt-pathway contributions in the developing vertebrate kidney may additionally be relevant to the generation of tubules in other organs and cell types.

1320

**Essential Function of FGF Signaling Pathway During Branching Morphogenesis of Mammary Gland**

P. Lu, A. Ewald, G. Martin, Z. Werb; Anatomy, UCSF, San Francisco, CA

Fibroblast growth factor (FGF) signaling plays an essential role in a variety of developmental processes and its deregulation is known to be responsible for many human malignancies, including breast cancer. Previously, it has been shown that FGF signaling is required for the formation of the mammary gland during embryonic development. However, its role in branching morphogenesis during postnatal mammary gland development has remained unclear. Using a combination of *in vivo* and novel three-dimensional (3D) *in vitro* culture systems, we have examined the role of FGF receptor 2 (FGFR2) in mammary gland branching morphogenesis. We found that *Fgfr2* was expressed in both the Cap Cells and the Body Cells of the terminal end buds at the invasion front, as well as in the luminal epithelium, but not the myoepithelium, of the trailing duct. Removal of *Fgfr2* function in the mammary epithelium using MMTV-Cre caused a severe retardation of branching phenotype in virgin females. By using different reporter lines in this genetic mosaic analysis, we found that epithelial cells lacking *Fgfr2* were out-competed by neighboring non-mutant cells and were absent from the invasion front. We are currently using a time-lapse confocal analysis in a 3D *in vitro* culture system to understand the cellular mechanism of how the competition occurs between *Fgfr2* null cells and non-mutant cells. Together, our results demonstrate FGFR2 is critically required for branching morphogenesis in mammary gland development.

## Cell Polarity II (1321-1336)

1321

### Role of p21-activated Kinases (PAK's) in Polarization in *Saccharomyces cerevisiae*

L. Kozubowski, T. Zyla, D. J. Lew; Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC

In *Saccharomyces cerevisiae*, the Rho GTPase Cdc42p is essential for cytoskeletal polarization. Normally, the site of polarization is dictated by a set of bud site selection proteins (including the Rsr1p GTPase and its GAP Bud2p). However, even in the absence of bud site selection components, cells polarize effectively to a single random site by a symmetry-breaking mechanism that requires the scaffold protein Bem1p. Cdc42p acts through many effectors, including p21-activated kinases (PAKs: Cla4p, Skm1p, Ste20p), which also bind to Bem1p. To test whether PAKs were collectively required for symmetry-breaking polarization, we asked whether PAK-less cells could polarize in the absence of Rsr1p or Bud2p. We found that in the absence of PAKs and Rsr1p cells could polarize secretion (assessed by Sec4p localization) and form abnormally shaped buds. Thus, unlike Bem1p PAKs are not required for symmetry-breaking polarization. Surprisingly, however, cells lacking PAKs and Bud2p (and therefore predicted to contain high levels of Rsr1p-GTP) failed to undergo sustained polarization. Similarly, PAK-less cells expressing a GTP-locked Rsr1p failed to undergo sustained polarization even though they had wild-type Bud2p. Thus, in the absence of PAKs excess Rsr1p-GTP impairs polarization. These observations are reminiscent of previous findings that *cln1Δ cln2Δ bud2Δ* strains are inviable whereas *cln1Δ cln2Δ rsr1Δ* strains are not (Benton B.K. *et al.*, 1993, EMBO vol. 12 no. 13 pp. 5267-75). Thus, it may be that the G1 cyclins Cln1p and Cln2p phosphorylate PAKs to promote sustained polarization, which is antagonized by excess Rsr1p-GTP.

1322

### Role of Protein Phosphatase Type 1 and Afr1 in Polarized Morphogenesis and Maintenance of Cell Integrity in *Saccharomyces cerevisiae*

J. P. Bharucha,[1] J. R. Larson,[1] J. B. Konopka,[2] K. Tatchell[1]; [1]Biochemistry and Molecular Biology, LSUHSC, Shreveport, LA, [2]Molecular Genetics and Microbiology, SUNY Stony Brook, Stony Brook, NY

Substrate specificity for type 1 protein phosphatase (PP1), a highly conserved phospho-serine/threonine phosphatase, is controlled in part by targeting subunits that direct PP1 to specific substrates. In the yeast *S. cerevisiae,* the catalytic subunit of PP1 (Glc7) is targeted to many subcellular locations in vegetatively growing cells and to the base of mating projections during the mating response. We show here that Afr1, a protein previously implicated in negative regulation of pheromone signaling and polarized morphogenesis, is responsible for the mating-specific targeting of Glc7. Glc7 fails to localize to mating projections in *AFR1* null mutants. Afr1 and Glc7 are shown to interact using FRET and two hybrid assays. It was shown previously that *AFR1* null mutants have aberrant mating projections. Afr1$^{V546A/F548A}$, a variant that fails to bind Glc7, also has aberrant mating projections, suggesting that Glc7 regulates cellular morphogenesis during mating. Chs4, an activator of chitin synthase 3, which is normally located at the necks of mating projections, accumulates at the tips of projections in *AFR1* null cells. Increased expression of *AFR1* results in increased accumulation of GFP-Chs4 throughout mating projections. The septin architecture in mating cells is also aberrant in *afr1* mutants. *AFR1* expression is induced during vegetative growth in response to cell wall perturbing agents such as Zymolyase that activate the Pkc1-Slt2 cell integrity signaling pathway. We show here that Afr1 protein also accumulates in response to Zymolyase. *AFR1* null and *afr1*$^{V546A/F548A}$ mutants are 10-fold more sensitive to Zymolyase than wild-type strains. Together, these results indicate that Afr1-Glc7 has roles in morphogenesis and/or cell wall synthesis during both mating and cell wall stress responses.

1323

### G Protein Regulation of Directional Sensing in Yeast

D. Suchkov, D. E. Stone; Laboratory for Molecular Biology, University of Illinois at Chicago, Chicago, IL

During the mating response of *S. cerevisiae,* cells are able to detect the pheromone gradient and polarize their growth toward their mating partners. It has been estimated that a 1% difference in receptor occupancy across the length of a cell leads to a correct orientation. It is presumed that a network of interacting feedback loops acts to sharpen the shallow attractant gradient, but the mechanisms responsible for this are not clear. We demonstrate that growth of mating projections is preceded by the formation of crescents of Ste2-GFP on the side of the cell facing the pheromone source. This redistribution of the receptor is the earliest known manifestation of directionality in the mating response. Mutations in *CDC24* and *CDC42* conferred a defect in, but did not abolish the formation of receptor crescents. Thus, polarized secretion is not essential for the onset of the ligand-induced redistribution of the receptor. Mutations in the receptor that prevent its internalization were found to prevent formation of a receptor crescent and confer a defect in partner discrimination. The same effects were observed in the cells lacking activity of Yck1 and Yck2 required for the receptor downregulation. Interstingly, we found that Yck1 interacts with Gβ genetically and binds to the Gβγ affinity matrix. Previously, Gα has been implicated in recruitment of MAPK Fus3 to the plasma membrane to phosphorylate cortical substrates. One of such substrate candidates is Gβ, which phosphorylated during the pheromone response. Gα mutant that prevents its interaction with Fus3 and a phosphorylation deficient allele of Gβ were found to negatively affect formation of receptor crescents. On the basis of these and other results, we propose a model in which G protein subunits regulate local feedback loops to promote redistribution of the receptor and regulate directional sensing.

1324

### Establishment of Polarized Cortical Domains Defining the Axes of Ascidians Oocytes and Embryos

C. Sardet, F. Prodon, P. Dru, A. Paix, J. Chenevert; Biologie du Developpement, CNRS, Villefranche sur Mer, France

To understand how the primary animal vegetal (a-v) axis is established we have analyzed oocytes at the Germinal Vesicle (GV) stage and spontaneously maturing (Stage4) oocytes of the ascidian *Ciona intestinalis*. We show that the subcortical mitochondria-rich myoplasm domain and a class of cortical maternal mRNAs (*postplasmic/PEM* RNAs) move into the oocyte periphery during oogenesis. Polarization along the a-v axis is initiated after GV BreakDown (GVBD) by the migration of the meiotic apparatus (MI) towards the egg cortex defining the animal pole. Within 2 hours of GVBD and spindle positioning, the myoplasm domain and cortical *Postplasmic/PEM* RNAs are excluded from the animal pole region. The primary a-v axis of the mature Stage4 oocyte in *Ciona, Phallusia* and *Halocynthia* is defined by these animal vegetal gradients of subcortical myoplasm distribution and of the underlying cortical domain made of a monolayer of cortical Endoplasmic Reticulum attached to the plasma membrane (cER). After fertilization some postplasmic/PEM RNAs (*PEM1, macho-1*) associated with the cER move with it in two major phases

successively driven by microfilaments and microtubules. These translocatios establish the Dorso Ventral (D-V) and Antero-Posterior (A-P) axes(see Prodon et al J. Cell Science 2005). In conclusion: the cortical cER/mRNA domain originally established along the a-v axis during oogenesis and maturation is compacted and relocalized into a posterior macroscopic cortical structure becoming a Centrosome Attracting Body (CAB). This CAB structure acts as an organizer in patterning the embryo along the A-P axis and in generating larger and smaller cells which are respectively the precursors of the muscle cells and of presumptive germ cells.

1325

**Establishment of Apical-Basolateral Cell Polarity at First Embryonic Division via Targeted Exocytosis is Maintained by the Actin Cytoskeleton**

M. Ng, D. Burgess; Biology, Boston College, Chestnut Hill, MA

One result of early cleavage divisions is formation of a simple epithelium composed of cells with distinct apical and basolateral surfaces. Such polarity is thought to be established after the first 3 or 4 divisions with the assembly of tight junctions. We find that early dividing sea urchin eggs establish and maintain distinct apical and basolateral surfaces in the absence of apical tight junctions by use of the actin cytoskeleton. Apical membranes were observed in early live sea urchin embryos using Alexa 488 cholera toxin subunit B (CTB), which binds to ganglioside GM1found in lipid rafts. The CTB label co-localized with integrins in fixed, immunostained embryos. Integrins were detected in isolated detergent resistant membranes, thought to represent lipid rafts. During cytokinesis of the first cell division, CTB was enriched in the cleavage furrow but cleared out from the membrane between the two daughter blastomeres at the end of cell division, due to new membrane addition via targeted exocytosis. In all embryos observed up to the 16 cell stage, the microvillus covered apical plasma membrane surfaces on the outside of the embryo were labeled with CTB whereas basolateral inner plasma membrane surfaces were not. When embryos were dissociated into single blastomeres at the four cell stage, the CTB labeled membrane domains remained polarized throughout the next cell cycle. Treatment of these dissociated blastomeres with Cytochalasin D, to depolymerize actin, disrupted these polarized membrane domains, and the CTB labeled membrane spread evenly along the whole surface of the cells. We conclude that apical and basolateral polarity is established in embryos as early as the two cell stage, well before tight junctions are formed, and that this membrane polarization is maintained by the actin cytoskeleton.

1326

**Investigation of the Cytoskeletal Mechanisms Required for Apical Constriction During *Xenopus* Gastrulation**

J. Lee, R. M. Harland; Dept of Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Cell shape changes are often required during morphogenesis to bend tissues and to shape organs. During *Xenopus laevis* gastrulation, bottle cells in the dorsal marginal zone (DMZ) are the first cells to undergo cell shape changes, transforming from cuboidal-shaped to flask-shaped. These cell shape changes create a crevice to direct the first steps of mesodermal involution. Apical constriction, a morphogenetic mechanism driven by polarized actomyosin contractile machinery, is hypothesized to cause bottle cell formation. Despite its importance during morphogenesis, little is understood about the mechanisms required for apical constriction. We are investigating apical constriction by examining cytoskeletal localization and dynamics during *Xenopus* bottle cell formation. Using fluorescence-conjugated phalloidin, we observed an intense accumulation of F-actin at the apical surface of bottle cells. Curiously, preliminary experiments with the microfilament inhibitors cytochalasin D and latrunculin A do not completely block apical constriction, but instead affect the directionality and pattern of constriction. Using time-lapse videomicroscopy, we also examined the behavior of bottle cells in explants cut lateral to the DMZ, in order to observe bottle cell formation as it happens. In unmanipulated embryos, bottle cells initiate as a small crescent in the central DMZ and spread laterally in both directions until the bottle cells form a complete circle (blastopore) in the vegetal pole of the embryo. In contrast, bottle cells in lateral explants initiate from either end and move towards the center. We are currently investigating whether these lateral bottle cells behave similarly to DMZ bottle cells. We are also assessing the role of microtubules and myosin during apical constriction by examining their localization and by perturbing their dynamics with pharmacological inhibitors. Through these experiments, we hope to gain further insight into the cytoskeletal mechanisms that control apical constriction.

1327

**Intrinsic Chiral Properties of the *Xenopus* Egg Cortex and Left-Right Asymmetry**

M. V. Danilchik, E. E. Brown; Integrative Biosciences, Oregon Health & Science Univ, Portland, OR

Background: A long-standing problem in the development of vertebrate left-right (LR) asymmetry is how an embryo's original bilateral symmetry becomes modified to initiate left- and right-specific regulatory pathways. In the large, cylindrically symmetric egg of *Xenopus laevis* , the earliest steps in dorsal-ventral (DV) axis determination are driven by microtubule-dependent rearrangements of localized maternal components toward the prospective dorsal side. Although LR axis determination is linked to this DV-determining localization process, the earliest steps are not understood. Results: Parthenogenetically activated eggs treated with millimolar concentrations of 2,3-butanedione monoxime undergo a dramatic large-scale counterclockwise torsion, with the cortex of the animal hemisphere shearing in a consistent direction past the vegetal cortex. Parallel microfilament bundles orient along the zone of maximal shear that develops near the egg's equator. Confocal time lapse of GFP-actin-expressing eggs undergoing torsion showed that the cortical torsion is accomplished by incremental shear between adjacent microfilament bundles. Thus, the large-scale torsion is microfilament-dependent. Cortical shear continues unabated following application of nocodazole, cytochalasin B, or jasplakinolide, but is halted by latrunculin B, indicating that the torsion is not organized by microtubules, and likely depends on preexisting f-actin fibers rather than new actin polymerization. Conclusions: A maternally inherited, microtubule-independent property of the *Xenopus* egg cortex is capable of consistently orienting microfilaments circumferentially -not radially- around the animal-vegetal axis, and therefore perpendicular to the embryo's midsagittal plane, regardless of the DV axis orientation. This unusual chiral arrangement may be important for cueing early cytoskeleton-dependent localizations in LR axis determination. Support: NSF IBN-0110985 and IBN-0419664

1328

**Determinants of *Drosophila* Non-muscle Myosin II Heavy Chain (Zipper) Cortical Localization**

R. R. Penkert, S. Liu, K. E. Prehoda; Molecular Biology, University of Oregon, Eugene, OR

The non-muscle myosin II is required for cytokinesis and cell-fate determinant segregation during asymmetric cell division in the *Drosophila*

neuroblast. We have sought to identify the components of Zipper required for cortical localization during asymmetric cell division. Myosin is a hexamer, which is composed of two regulatory light chains (Spaghetti Squash), two essential light chain and two heavy chains (Zipper). Zipper has an N-terminal actin binding head and a long coiled-coil tail. Myosin II monomers are known to assemble into bipolar thick filaments through intermolecular interactions between heavy chain tail domains. Various truncations of the Zipper tail were made and cloned into bacteria and insect cell expression vectors. The purified truncated proteins were analyzed by Circular Dichroism (CD) and Analytic Ultracentrifugation (AUC) to determine folding, stability and oligomerization state. *Drosophila* S2 cells were transiently transfected with these Zipper fragments and localization was determined by fluorescent immunostaining and image analysis. The tail region of Zipper is sufficient for localization but fragments that are unable to form thick filaments lack the ability to properly localize. This suggests that filament formation may be a prerequisite for tail localization and not interactions of the head domain with actin filaments.

1329

**An Intramolecular Interaction within the Cell Polarity Protein Pins Cooperatively Represses Gα Binding at two Internal GoLoco Domains**

R. W. Nipper, K. E. Prehoda; Institute of Molecular Biology, University of Oregon, Eugene, OR

The *Drosophila* cell polarity protein Partner of Inscuteable (Pins) and the heterotrimeric G protein subunit, Gα, are essential components in neuroblast asymmetric division. G proteins are important in asymmetric division, as Gα and Gβγ downstream effectors modulate microtubule dynamics, potentially regulating spindle formation in neuroblasts. Pins dissociates G protein complexes at the apical cortex using three carboxyl terminal GoLoco domains which each sequester Gα subunits. Recent work has described an intramolecular interaction between the N and C terminus of the mammalian Pins homologue, LGN. Here we report Pins undergoes a similar intramolecular interaction between its N-terminal tetratricopeptide repeats (TPR) and C-terminal GoLoco array. As previously reported, this autoregulatory interaction represses overall Gα binding to the GoLoco domains. Here, we further refine the model of the intramolecular interaction within the *Drosophila* Pins protein. We have found the TPR domains differentially regulate the three GoLoco domains within Pins. The TPR region cooperatively represses the two GoLoco domains at the carboxyl terminus, but does not affect the most amino-terminal GoLoco. We propose Pins can bind a basal quantity of Gα though the unaffected GoLoco, even in a closed conformational state, while relief of the intramolecular interaction increases the Gα binding capacity of the GoLoco array. We postulate that differential Gα regulation in Pins may allow for noise suppression and cooperativity in cell polarity signaling.

1330

**Expression and Function of *Fat* Cadherins within Developing Inner Ear**

M. R. Deans, A. Tucker, L. V. Goodrich; Neurobiology, Harvard Medical School, Boston, MA

Mammalian *fat* cadherin genes encode unusually large transmembrane proteins containing 34 cadherin repeats. In *Drosophila*, *fat* regulates planar cell polarity, as well as imaginal disc growth and patterning. Development of the mouse inner ear involves similar processes including the polarization of hair cells within the plane of the sensory epithelia, and generation of the correct numbers and ratio of hair cells and supporting cells. We hypothesize that a conserved Fat pathway regulates some, if not all, of these events. To explore the function of mammalian *fats* we have mapped the distribution of all four *fat* homologs within the developing ear and evaluated the function of *fat1* in mutant mice. *In-situ* hybridization and x-gal staining in transgenic mice indicate *fat1* expression throughout the otocyst at embryonic day 10 (E10) and subsequent restriction to support cells and non-sensory regions by E18. In contrast, *fat3* is present transiently between E14 and P0 in developing sensory epithelia in a pattern similar to the hair cell transcription factor *math1*. *Fat2* and *fat4* are not expressed in the cochlea. The GST070 mouse line carries a gene trap insertion that disrupts *fat1* function. Due to the significance of *fat* in planar cell polarity in flies, we evaluated the $fat1^{GST070}$ mutant mice for hair cell polarity defects by phalloidin and tubulin labeling of whole mount organ of Corti. No polarity defects were seen for inner or outer hair cells. Therefore, based upon gene expression data and mutant analysis, we propose *fat3* as a candidate regulator of planar cell polarity within the developing ear, and are testing this model by generating *fat3* mutant mice.

1331

**The Role of Cdc-42 And Rho-1 in Establishment of Polarity in *C Elegans* Embryos**

S. Schonegg, A. Hyman; MPI-CBG, Dresden, Germany

In the *C. elegans* one-cell embryo the establishment of anterior-posterior axis is marked by the segregation of partioning-defective (PAR) proteins into anterior (PAR-3, PAR-6) and posterior (PAR-1, PAR-2) cortical domains. Concomitantly with establishment of PAR polarity, the embryo undergoes cortical rearrangements leading to differences in contractile activity of the cortex along anterior-posterior axis (contractile polarity). The underlying mechanism of cortical dynamics and its relationship to PAR polarity is unclear. To investigate how modulation of the acto-myosin cytoskeleton affects PAR polarity establishment, we perturbed the acto-myosin cytoskeleton by RNAi of members of the Rho GTPase family, CDC-42, RHO-1 and ECT-2 and analyzed PAR protein distribution and the organization and dynamics of nonmuscle myosin 2 (NMY-2). We show that they are involved in different ways in establishment of polarity. CDC-42 and RHO-1 are required for PAR localization and in particular, RHO-1 is required through the organization of the acto-myosin cytoskeleton to establish the boundary between the anterior and posterior PAR proteins.

1332

**W08f48, Encoding the *C Elegans* Homologue of Cdc37, Plays a Role in Establishing Early Embryo Polarity**

M. A. Beers, K. J. Kemphues; Molecular Biology & Genetics, Cornell University, Ithaca, NY

Establishment and maintenance of cellular asymmetry and polarity is a hallmark of the development of many multicellular organisms. In *C. elegans*, polarity established in the one cell embryo determines the anterior-posterior axis and the identity of the early blastomeres. The *par* genes (*par*titioning defective) are maternal-effect lethal genes that when mutated disrupt polarity in the early embryo. PAR-3, PAR-6 and PKC-3 proteins colocalize at the anterior cortex of the one cell embryo while PAR-1 and PAR-2 proteins colocalize at the posterior cortex. These two protein domains are maintained by mutual exclusion. It has also been shown that the presence of PAR-6 and PKC-3 at the cortex requires PAR-3. We and others have identified W08F4.8, the *C. elegans* homologue of CDC37, as a gene with a role in early embryo polarity. My goal is to determine: 1) in what stage of polarity establishment W08F4.8 participates, 2) where it is located in the cell and 3) how it functions. To address these questions we

have used a combination of RNAi, antibody staining and analysis of different GFP expression lines. My work shows that W08F4.8 RNAi embryos exhibit all of the phenotypes characteristic of *par* mutants. Unlike many of the PAR proteins, a W08F4.8::GFP fusion protein is found in the cytoplasm throughout most of the cell cycle. At nuclear envelope breakdown it becomes enriched in the nucleoplasm and then localizes to the central spindle of the dividing cell. We found that W08F4.8 functions in the establishment phase of polarity determination. Most interestingly, we learned that the reduction of W08F4.8 by RNAi can uncouple the mutual exclusion of the anterior and posterior PAR's and also uncouples the cortical localization of PAR-6 and PKC-3 from their dependence on PAR-3.

1333

**Role of the Actin Cytoskeleton in Early *C elegans* Development**

N. Velarde, F. Piano; Biology, New York University, New York, NY

Early *C. elegans* embryonic development requires key events that involve cortical interactions with the cytoskeleton. I am interested in studying the role of F-actin in the early embryo. To visualize F-actin dynamics *in vivo*, I have generated a transgenic line that uses the F-actin binding domain of *Drosophila* moesin to decorate endogenous F-actin with GFP (PF100). This line appears to be specific for F-actin and allows us to faithfully visualize actin dynamics in *C. elegans*. Using timelapse microscopy, I have been able to gather preliminary evidence that suggests that the cytoskeleton is playing key roles throughout early development of the *C. elegans* zygote. The time periods I have focused on are fertilization and early embryogenesis. Right after fertilization, a transient "actin cap" appears on the presumptive posterior and then dissipates, which may be an important initial step for generating polarity. Prior to the first cell division F-actin becomes enriched in the anterior, similar to the anterior PAR proteins. As seen in *par-3* mutants (Kirby et. al., 1990), depleting the embryo of PAR-6 with RNAi prevents this enrichment, suggesting that PAR-6 and the other anterior PARs may be required for F-actin to accumulate in the anterior. Other known regulators of the PAR protein network also affect the collapse of the F-actin network to anterior half of the embryo. We have also observed the presence of highly dynamic actin comets in the early embryo. Preliminary evidence suggests that these comets may be involved with vesicle or organelle trafficking in the cell. We will present our progress in using this GFP::Moe line to study genetic interactions involving actin dynamics in early development.

1334

**Rotation of 1-cell C elegans Embryos Inside the Egg Shell Reveals an Early Step in Establishment of Left-right Polarity and Embryonic Handedness**

S. Schonegg,[1] A. A. Hyman,[1] W. B. Wood[2,1]; [1]Max Planck Institute for Cell Biology and Genetics, Dresden, Germany, [2]MCD Biology, Univ. of Colorado, Boulder, Boulder, CO

The initial symmetry-breaking mechanism for establishing left-right (L-R) asymmetry and handedness is not understood in any embryo. C. elegans embryos first exhibit obvious L-R asymmetry at the 6-cell stage; however, asymmetry can already be seen during second cleavage, when the mid-body between the two AB daughter cells invariably moves to the right, apparently due to asymmetric closure of the cleavage furrow. This suggests a pre-existing L-R asymmetry in the AB-cell cortex at the 2-cell stage. In searching for possible earlier asymmetry cues, we observed that just prior to initiation of first cleavage, the entire embryo rotates for ~50 sec, through an angle of ~120Â°, inside the vitelline membrane of the egg shell. The screw sense of the rotation is invariant relative to the already established anterior-posterior (A-P) polarity, indicating that the 1-cell embryo has an intrinsic chirality. Strong maternal knock-down (KD) of gene products required for actomyosin function or microtubule formation block the rotation. KD of PAR-2, PAR-3, PAR-6, and other gene products required for establishment and maintenance of early A-P polarity results in partial randomization of the direction and extent of rotation as well as the handedness of the later embryo. These and additional KD experiments indicate that normal chirality of the 1-cell embryo 1) requires integrity of A-P polarity and 2) may be an initial cue for establishment of L-R polarity with correct choice of handedness.

1335

**Mosaic Eyes Protein Forms a Complex and Colocalizes with Homologues of Crumbs and Pals1, and aPKC**

A. M. Jensen,[1,2] Y. Hsu,[1,2] J. Willoughby,[1] A. Christensen[1,2]; [1]Biology, University of Massachusetts, Amherst, MA, [2]Program for Molecular and Cell Biology, University of Massachusetts, Amherst, MA

Previously, we identified the *mosaic eyes* (*moe*) gene that encodes a FERM protein and is required for retinal lamination in zebrafish. Expression of *moe* in photoreceptors suggests that *moe* may also play a role in photoreceptor differentiation or morphogenesis. We sought to determine whether Moe binds to the intracellular domain of vertebrate homologues of crumbs, all of which contain a FERM-binding motif. We first cloned zebrafish *crb1*, *crb2a*, *crb2b*, and *crb3b*, and we found an EST for *crb3a*; we examined their expression in the retina using *in situ* hybridization and observed expression of *crb2a* and *crb2b* in the retina. We made fusion proteins of Moe and crumbs homologue proteins for biochemical analysis. We found that HIS-Moe_FERM pulls-down a protein(s) from lysates that is immunoreactive for anti-CRB3 (by Western, anti-CRB3 recognizes all zebrafish crumbs homologue proteins examined) with a molecular weight predicted for Crb2a and Crb2b. Immobilized HIS-Moe_FERM pulls down GST-tagged intracellular domains of Crumbs homologues and HIS-Moe_FERM immobilized on nitrocellulose binds GST-Crb1$^{intra}$ and GST-Crb2a$^{intra}$. In addition, Moe forms a complex with Nagie oko (Pals1 homologue) and aPKC. We show that anti-Moe and anti-CRB3 immunoreactivity colocalize in photoreceptors. There are two major splice variants of Moe in vertebrates, a long and short isoform; the proteins differ after the FERM domain. Previously we described the long isoform; the mouse short isoform removes about 250 amino acids and adds about 50 unique amino acids at the C' terminus. Mouse photoreceptors express both isoforms, but not all cell types do so. We are examining the roles of the long and short isoforms in potentially regulating crumbs homologue function during the process of light-regulated photoreceptor outer-segment renewal. Preliminary chimera/transplant experiments suggest *moe* is required for normal photoreceptor morphology.

1336

**Regulation of Mosaic Eyes by Calmodulin and Photoperiod in Zebrafish Photoreceptors**

A. Christensen,[1,2] J. Willoughby,[1] Y. Hsu,[1,2] A. Jensen[1,2]; [1]Biology Department, University of Massachusetts, Amherst, MA, [2]MCB Program, University of Massachusetts, Amherst, MA

Mosaic eyes (Moe) is a Band 4.1 family member with significant homology within the FERM domain to other family members. During

development, Moe is required for proper retinal, brain, and kidney morphogenesis. Our lab has shown that Moe forms a complex with homologues of Crumbs and Pals1 proteins and may play a role in photoreceptor morphogenesis. Using immunohistochemistry, we show that Moe colocalizes with Crumbs homologues, Nagie Oko (Pals1 homologue), aPKC and Calmodulin in photoreceptors. In order to further elucidate the role of Moe in photoreceptors, we are examining its regulation. We show that the FERM domain of Moe binds Calmodulin. Western blot analysis of adult retinas with anti-Moe antibody reveals two immunoreactive bands, migrating with molecular weights of 110 and 55 KDa. Further, the expression of each Moe species is photoresponsive; the large Moe band, but not the small, is present during dark periods, whereas exposure to light causes the appearance of the small Moe band and disappearance of the large. There are two major splice variants of Moe in vertebrates, a long and short isoform, which may encode the 110 and 55 KDa proteins observed. The isoforms differ after the FERM domain. Mouse photoreceptors co-express both isoform mRNAs, although not all cell types co-express both. The light/dark-induced shift in molecular weight species possibly reflects a process of selective protein degradation. Based on these data, we hypothesize that the long Moe isoform negatively regulates Crumbs and that a shift to the smaller isoform facilitates the process of light-regulated photoreceptor outer-segment renewal and this regulation depends on exogenous light/dark cues and intracellular calcium levels mediated by Calmodulin. Our observations may offer the first molecular handle on the hitherto unknown mechanism that controls the important process of photoreceptor outer-segment renewal.

## Development & Carcinogenesis (1337-1342)

1337

### Notch Target Genes Bind to and Modulate Function of the Pancreatic Transcription Factor, Ptf1-p48

B. Ghosh, S. D. Leach; Surgery, John Hopkins University School of Medicine, Baltimore, MD

Notch signaling has been shown to have a normal inhibitory effect in the regulation of exocrine differentiation, mediated at least in part by direct inhibition of the pancreatic transcription factor Ptf1-p48. To further elucidate the biochemical mechanism by which Notch inhibits Ptf1 function, we have designed experiments to test the hypothesis that the Notch target genes Hes1, Hey1 and Hey2 can physically interact with Ptf1-p48 to form inactive heterodimers. We have also systematically analyzed the different domain structures of both Ptf1-p48 as well as Hes1, in order to identify specific domains required for this interaction. Co-immunoprecipation analysis in COS7 cells and GST pull down assays indicated that Ptf1-p48 formed a protein complex with each of the three Notch target genes. Evaluation of GST fusion proteins encoding various Ptf1-p48 deletion mutants revealed that only full length Ptf1-p48 was able to bind Hes1. Conversely, deletion mutant analysis of Hes1 indicated that the carboxyl terminal and basic helix-loop-helix domains were necessary for Ptf1-p48 binding. We also confirmed physical interaction between Ptf1-p48 and Hes1 through a yeast two-hybrid approach, using the carboxyl terminal and basic HLH domain of Ptf1-p48 as bait. Finally, in transient transfection experiments using a Ptf1-responsive luciferase reporter in the presence of Ptf1, E47 and different deletion mutants of Hes1, we found significant inhibition of Ptf1 function only in the presence of Hes1 deletion mutants containing both the basic HLH domain and the carboxyl terminal, demonstrating a direct correlation between Hes1's ability to bind Ptf1-p48 and its ability to inhibit Ptf1 function. Taken together, these data provide a mechanism by which Notch and its target genes repress epithelial differentiation in exocrine pancreas, an effect likely to play an important role in the pathogenesis of human pancreatic cancer.

1338

### Implication of Lagy in Lung Carcinogenesis and Development

Y. Chen, I. Petersen; Institute of Pathology, University Clinic Charité, Berlin, Germany

We have isolated a novel human lung cancer-associated gene, LAGY (lung cancer-associated Y protein) by suppression subtractive hybridization (Chen Y. et al., PMID 15386363). The nucleotide sequence of LAGY predicts a small protein of 73 amino acid containing a putative Homeobox domain with molecular mass of 8.1 kDa. Multiple tissue Northern blots analysis revealed that the gene is present in human placenta, lung, brain, heart and skeletal muscle. Gene mapping locates LAGY gene on chromosome 4q11-13.1. The expression of LAGY mRNA levels was widely lost in 5 immortalized human bronchial epithelial cell lines and 18 lung tumor cell lines comprising 4 major histological types including small cell lung carcinoma (SCLC), large cell lung carcinoma (LCLC), squamous cell carcinoma (SCC) and adenocarcinoma (ADC) by Northern blot analysis and semi-quantitative reverse transcription-polymerase chain reaction (RT-RCR). In the survey of 72 primary lung tumors (37 ADCs, 27 SCCs, 4 SCLCs and 4 LCLCs), this gene was significantly down-regulated in all tumors compared to 9 normal lung tissue samples, most prominently in SCC. For this tumor type, there was also a significant reduction for LAGY expression in tumors with increased grade and stage. No expression was detectable in two high grade SCC and 50% of SCLC and LCLC, respectively. Since homeodomain-containing genes are known to transcriptionally regulate key cellular processes and have been associated with carcinogenesis, we suggest that the LAGY gene should be considered as a candidate tumor suppressor in lung cancer development and progression. Furthermore it has meanwhile been shown to be of key importance in cardiac development (Chen F. et al., PMID 12297045).

1339

### Myc-Mediated Regulation of Ephs During Cancer and Development

B. Yekkala, T. A. Baudino; Cell and Developmental Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, SC

Eph receptor tyrosine kinases and their cognate ligands ephrins, participate in numerous developmental processes such as embryonic patterning, axonal guidance and angiogenesis. Additionally, their overexpression has been shown to promote metastases by inducing tumor growth and angiogenesis in many human cancers including colon, prostate and breast. However, the precise mechanism(s) involved in Eph and ephrin regulation remain to be elucidated. The Myc family of basic helix-loop-helix (bHLH) leucine zipper transcription factors (c-Myc, N-Myc and L-Myc) regulate a wide range of cellular processes including proliferation, apoptosis, vasculogenesis and hematopoiesis. Recent studies from our laboratory have demonstrated the regulation of Vascular Endothelial Growth Factor (VEGF) and other angiogenic factors by c-Myc. These data suggest that Myc acts as an angiogenic switch during cancer and development. Therefore, we hypothesized that Myc may also regulate Eph receptor family members. We have investigated this hypothesis by examining Eph gene expression using both *in vitro* and *in vivo* techniques. Using primary mouse embryonic fibroblasts (MEFs) expressing an inducible Myc-ER fusion protein, we examined the expression of all known Eph receptors. Following activation of Myc-ER with 4-Hydroxytamoxifen, we identified *EphB4* and *EphA2* as target genes for c-Myc. We have

selected *EphA2* as our candidate gene for further studies since it has been shown to be dysregulated in many cancers. Our studies suggest that Myc-mediated regulation of *EphA2* is direct, while regulation of *EphB4* is indirect. Moreover, c-Myc activation induces robust *EphA2* promoter activity in MEFs. Furthermore, loss of c-Myc results in a significant reduction in *EphA2* expression in $APC^{Min}$ intestinal polyps. Finally, c-Myc loss *in utero* results in reduced levels of *EphA2.* Taken together, these data suggest that c-Myc plays a critical role in the transcriptional regulation of specific Ephs.

1340

**Chromium Exposure Leads to Chromosome Instability in *C elegans***

A. Sood,[1] J. W. Astin,[2] P. E. Kuwabara,[2] C. P. Case[1]; [1]Dpt. of Orthopaedic Surgery, University of Bristol, Bristol, United Kingdom, [2]Dpt. of Biochemistry, University of Bristol, Bristol, United Kingdom

Humans are exposed to chromium from many sources: industry, the environment and cobalt chrome alloys in surgical implants. Hexavalent chromium is a complex genotoxin causing multiple lesions, including single- and double-strand DNA breaks, oxidative base damage, DNA-DNA cross-links, DNA-protein cross-links and Cr-DNA adducts. It has been established that chromium can initiate the formation of reactive oxygen species through the reduction of Cr(VI) to Cr(III). To understand the basis of chromium genotoxicity *in vivo*, we are using the model organism *Caenorhabditis elegans. C. elegans* is an established model for studying human disease; homologues to over 70% of known human disease genes are present in the genome of *C. elegans.* Dose response studies using the metallic salts potassium dichromate ($K_2Cr_2O_7$) have been performed. Based on these results, a range of concentrations have been tested to determine the sensitivity of the germ line to hexavalent chromium by examining three parameters: brood size, germ line apoptosis and oocyte chromosome number. We find that wildtype animals are sensitive to chromium and display a decrease in brood size and an increase in chromosomal abnormalities, marked by a decrease in the number of bivalents during diakinesis. Animals defective in nucleotide excision repair, such as *xpa-1*, or that lack the checkpoint kinase, *atm-1,* also display chromium hypersensitivity. These animals have reduced brood sizes and an increase in chromosomal abnormalities when compared to wildtype. It is evident that chromium has an adverse effect on *C. elegans* development. Chromosomal abnormalities detected in oocytes are likely to be caused by chromosomal fusions; deletions are unlikely as embryonic lethality is low. We are presently examining whether the chromosomal abnormalities caused by chromium exposure are caused by end-to-end chromosomal fusions. In addition, we are investigating whether chromium exposure also leads to transgenerational chromosomal instability.

1341

**Dopamine Modulation of Cell Migration**

H. O. Lawal,[1] A. Hsouna,[2] T. Hsu,[2] J. M. O'Donnell[1]; [1]Biological Sciences, University of Alabama, Tuscaloosa, AL, [2]Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC

The tracheal development in *Drosophila* has been investigated in detail and is considered to be a model for mammalian angiogenesis since these processes share many gene product requirements. Tracheal cells migrate in response to FGF chemotactic signaling to form the branched tubular network, which delivers oxygen throughout the insect. However, the underlying mechanism of FGF receptor (FGFR) regulation has not been fully elucidated. We report the novel result that three interacting genes in the dopamine synthesis pathway, two that positively regulate and one that negatively regulates dopamine levels, appear to modulate FGFR levels during tracheal development. . Mutants in each of these genes produce tracheal phenotypes characteristic of a perturbation in FGF signaling. Using drugs to modulate dopamine levels in vivo, we have reproduced the tracheal phenotypes of the mutants. Embryos exposed to excess exogenous dopamine exhibit blocked tracheal cell migration, similar to those mutant for the negative regulator, which have elevated dopamine, while pharmacological depletion of endogenous dopamine causes ectopic cell migration, as observed in low dopamine mutant embryos. Migration of primary human endothelial cell can be similarly modified by dopamine modulation. Dopamine may be regulating FGF signaling by down-regulating the FGFR levels through endocytosis. Using an FGFR-GFP chimeric protein, we observe that FGFR, which is normally localized at the plasma membrane, is found in cytoplasmic compartments in embryos exposed to dopamine, while the receptor accumulates at the plasma membrane in dopamine-depleted embryos. Using pharmacological agonists and antagonists of dopamine receptor signaling, we show that dopamine may be acting through its receptors to modulate FGF levels. Taken together, our results suggest that dopamine acts through its receptor to regulate cell motility by modulating the FGFR levels.

1342

**The Role of LIM Proteins Ajuba and LIMD1 in the Nucleus**

E. M. Langer,[1] Y. Feng,[1] K. L. Kroll,[2] G. D. Longmore[1]; [1]Medicine and Cell Biology, Washington University School of Medicine, Saint Louis, MO, [2]Molecular Biology and Pharmacology, Washington University School of Medicine, Saint Louis, MO

Ajuba and LIM domains-containing protein 1 (LIMD1) are members of the cytosolic family of LIM domain-containing proteins that localize to sites of cell-cell and cell-matrix adhesions in epithelial cells and fibroblasts, respectively. Both of these proteins also shuttle to the nucleus, but little is known about the mechanisms by which they translocate to the nucleus, the identity of their nuclear targets, or the signaling pathway(s) they affect. The LIM region of Ajuba, which localizes to the nucleus, was used as bait in a yeast two-hybrid screen to identify the protein Snail as an interacting partner of Ajuba. Further analysis by co-immunoprecipitation confirmed that Ajuba interacts with Snail and its family member Slug. Snail and Slug are transcriptional repressors that mediate epithelial mesenchymal transitions in physiological processes such as gastrulation and neural crest delamination as well as in pathological processes such as cancer metastasis. In transient luciferase assays, Ajuba appears to cooperate with Snail in transcriptional repression of the E-cadherin promoter. In order to determine if this interaction is functional, we have used the *Xenopus laevis* model system in which Snail family members have well-described roles in neural crest specification and migration. To date, there appears to be only one Ajuba-like ortholog in *Xenopus*, which is most similar to LIMD1. We cloned the *X. laevis* ortholog of LIMD1 and detected its endogenous expression in developing embryos through *in situ* hybridization. Expression of LIMD1 overlaps with that of Snail and Slug, particularly in the neural crest. Microinjection of LIMD1 mRNA into embryos results in an expansion of the neural crest area, mimicking the effect of Slug overexpression. These results suggest that Ajuba and LIMD1, through their interaction with Snail family members, may play an important role in epithelial mesenchymal transitions.

## Synapse Formation & Function I (1343-1359)

1343

### Function of α-Dystrobrevin Isoforms in AChR Cluster Formation and Stabilization

B. T. Pawlikowski, M. M. Maimone; Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

In skeletal muscle, α-dystrobrevin (αDB) is expressed throughout the sarcolemma with high concentrations at the neuromuscular junction (NMJ). Alternative splicing generates three major αDB isoforms in adult (αDB1, αDB2, αDB3) and two more in early development (αDB1(-), αDB2(-)). Mice lacking αDB display multiple abnormalities including muscular dystrophy and NMJ defects that suggest a role for αDB in maturation of acetylcholine receptor (AChR) clusters. At adult NMJs, AChR clusters have a complex pretzel-shaped architecture which can be recapitulated in muscle cell culture in the absence of nerve or agrin. Using a primary muscle culture system generated from αDB knockout (αDB-KO) mice, we investigated the role of αDB in the formation of complex agrin-independent AChR clusters. In myotubes lacking αDB, complex AChR clusters were not observed, although they readily formed in wildtype myotubes. To determine which αDB isoforms could mediate formation of complex AChR clusters, individual αDB isoforms were expressed in αDB-KO myotubes using retroviral infection. Expression of αDB1 or αDB1(-) individually led to formation of complex AChR clusters similar to those observed in wildtype cells, whereas expression of αDB2, αDB2(-) or αDB3 individually resulted in few poorly developed clusters. These data suggest that αDB plays a role in the formation of complex AChR clusters and that protein domains uniquely present in αDB1 isoforms mediate this function. Similar analysis of agrin-induced AChR clusters, which are much less complex, showed that these clusters are less stable in the absence of αDB and that individual expression of αDB1, αDB1(-) or αDB2 (but not αDB2(-) or αDB3) could restore the stability to wildtype levels. Taken together these data demonstrate differential functions of individual αDB isoforms in the formation and maintenance of AChR clusters and suggest roles for specific protein domains of αDB.

1344

### Agrin and Laminin Induce Acetylcholine Receptor Clustering by Convergent, Rho GTPase-Dependent Signaling Pathways

G. Teressa, C. A. Weston, P. Hallock, J. Prives; Pharmacological Sciences, Stony Brook University, Stony Brook, NY

Under physiological conditions cell signaling is typically a complex phenomenon involving simultaneous utilization of several signaling pathways. During neuromuscular junction formation several identified molecular factors are utilized to trigger aggregation of muscle cell surface acetylcholine receptors (AChRs) adjacent to nerve endings. Two extracellular matrix proteins found in synaptic clefts, neural agrin and laminin, are each known to induce clustering of AChRs on the surface of cultured muscle cells by activation of separate transmembrane signaling mechanisms. To characterize the signaling pathways that regulate these aspects of synaptogenesis, we have compared the molecular events that couple agrin and laminin signaling to AChR clustering in C2 muscle cell cultures. Clustering of AChR was induced by overnight exposure of myotubes to soluble agrin, soluble laminin, or substrate bound laminin. The AChR clusters displayed characteristically different morphologies in each case, suggesting underlying differences in the clustering process or in the endpoint reached. In contrast to laminin, agrin-induced clustering was accompanied by increased tyrosine phosphorylation of AChR β subunits. Substrate-coated laminin induced clusters were unique in size, complexity and in showing co-localization with phosphorylated FAK. However, in all cases AChR clustering was accompanied by, and dependent on activation of the Rho GTPase signaling pathway. Thus, agrin, soluble laminin and substrate-coated laminin induced activation of Rac, and Rho, as measured with biochemical assays. Moreover, AChR clustering by these stimuli was abolished by dominant negative Rac and Rho mutants, as well as the Rho kinase inhibitor Y-27632. These findings provide evidence that the agrin and laminin signaling pathways converge such that these signals are coupled to AChR clustering by the actions of Rac, Rho, and Rho kinase. l

1345

### The β-subunit Intracellular Loop is Sufficient for Postsynaptic Clustering of the nAChR

S. Yechikhov, L. S. Borges, Y. I. Lee, M. Ferns; Physiology and Membrane Biology, UC Davis, Davis, CA

At the developing vertebrate neuromuscular junction, postsynaptic localization of the nAChR is regulated by agrin-MuSK signaling and requires an intracellular, membrane-tethered protein called rapsyn. However, the intracellular domains of the nAChR that interact with scaffolding proteins to mediate clustering are unknown, and it is unclear how such interactions are regulated by agrin. To define the subunit(s) and domains that mediate postsynaptic clustering of the AChR, we generated chimeric proteins consisting of CD4 extracellular and transmembrane domains fused to each of the AChR subunit large intracellular loops. When expressed in cultured muscle cells, CD4 and all the CD4- subunit chimeras were expressed on the myotube surface, but strikingly, only CD4-β loop coclustered with the endogenous AChR in response to agrin treatment. To map the domain mediating localization of the β loop, we tested a series of CD4-β loop deletion mutants and found that a 37 amino-acid sequence (aa 370-406) was sufficient for agrin-induced clustering. This sequence contains a conserved tyrosine (Y390) previously implicated in AChR clustering, and indeed, we found that agrin treatment induced rapid phosphorylation of Y390 in CD4-β loop chimeras and mutation of this residue completely abolished clustering. Finally, we found CD4-β 370-406 acted in a dominant-negative fashion when overexpressed in myotubes, inhibiting agrin-induced aggregation of the endogenous AChR by ~25%. Together, these results identify a domain specifically in the β subunit loop that is sufficient for clustering. Moreover, they suggest that protein interactions with this domain contribute to postsynaptic localization of the nAChR and that this is regulated by agrin-induced phosphorylation.

1346

### Effect of siRNA-Mediated Inhibition of APC on Agrin-induced Acetylcholine Receptor Clustering in C2C12 myotubes

Y. Yao,[1,2] L. Zhang,[2] Z. Wang[1,2]; [1]Department of Cell and Neurobiology, Zilkha Neurogenetic Institute, University of Southern California Keck School of Medicine, Los Angeles, CA, [2]Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Synaptogenesis at the neuromuscular junction (NMJ) is characterized by the aggregation of nicotinic acetylcholine receptors (AChRs) into high-density clusters on the postsynaptic muscle membrane. The signaling mechanisms that trigger AChR cluster formation and the cytoskeletal elements involved in immobilizing the receptors remain poorly understood. In a previous study, we have shown that adenomatous polyposis coli (APC) binds directly to the beta-subunit of muscle AChR in yeast two-hybrid and *in vitro* binding assays. APC is colocalized with AChRs in the mouse NMJ. The present study was undertaken to determine whether APC is required for the expression and agrin-induced clustering of AChRs in

muscle cells. C2C12 myocytes were infected with adenovirus expressing siRNA directed against APC. The green fluorescent protein (GFP) was co-expressed to allow easy identification of cells infected with the viral vector. The majority (>95%) of C2C12 myotubes were GFP-positive at 3 days post-infection. Immunoblotting of detergent extracts of the myotubes revealed a striking reduction (~82%) in the levels of the targeted protein APC. Immunofluorescent staining showed that inhibition of APC prevented the formation of large AChR clusters on the surface of myotubes exposed to neural agrin. Moreover, the levels of AChRs on myotubes surface were also reduced as determined by $^{125}$I-alpha-bungarotoxin binding assay. Northern blotting indicated that the levels of messenger RNAs for AChR subunits were not altered. In contrast, no change in APC protein levels, AChR clustering and expression was observed in C2 cells infected with adenovirus expressing siRNA directed against GAPDH. Our results suggest that APC may play a role in agrin-induced AChR clustering as well as trafficking or stability of AChRs in skeletal muscle cells. (Supported by NIH grant RO1-NS38301 and the Muscular Dystrophy Association).

1347

### Isolation and Characterisation of GluR1-Containing Clathrin Coated Vesicles

P. G. R. Hastie,[1] K. J. Heesom,[2] S. R. White,[1] B. Costley-White,[1] G. Banting,[2] J. M. Henley[1]; [1]MRC Centre for Synaptic Plasticity, University of Bristol, Bristol, United Kingdom, [2]Department of Biochemistry, University of Bristol, Bristol, United Kingdom

Objective: To identify proteins co-transported with GluR1 containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. It is believed that AMPA receptors go through both constitutive and activity dependent cycles of exo- and endocytosis in a clathrin-dependent manner. We provide further evidence for this by using a dominant negative mutant of AP180, a component of the clathrin coat machinery, to block AMPA receptor endocytosis in cell lines. However, vesicles containing AMPA receptors have not been biochemically isolated. We therefore devised a method to capture GluR1-containing clathrin coated vesicles (CCVs) from whole pig brain by immunoaffinity isolation. The advantage of enriching these vesicles over whole cell studies is that it is possible to identify novel proteins involved in AMPA receptor trafficking. These may not directly interact with the receptor or may be unknown proteins expressed at levels below normal detection thresholds. The proteomic content of these vesicles was therefore analysed by 2D SDS-PAGE and mass spectoscopy. This approach identified a number of hits including some uncharacterised proteins. These mostly lacked membrane association domains and modifications and may be trafficked in the vesicle lumen. AMPA receptors may be specifically selected as cargo to be incorporated into a CCV or be incorporated with other proteins which are then returned to the source membrane by retrograde transport. Immunoblot analysis suggests that NR1-containing N-methyl D-aspartate (NMDA) receptors and GluK6/7-containing kainate receptors are not co-transported with GluR1-containing AMPA receptors. These results favour a model of positive selection, implying some form of physical segregation of AMPA receptors from other membrane proteins. Interactions with the postsynaptic density are unlikely to be involved as CCVs form at extrasynaptic sites although there may be a role for membrane microdomains, eg lipid rafts.

1348

### Analysis of the Synaptic Vesicle Proteome: Pros and Cons Using Three Different Gel Separation Techniques in Combination with Mass Spectrometry

J. Burré,[1] H. Schägger,[2] T. Beckhaus,[3] C. Corvey,[3] M. Karas,[3] H. Zimmermann,[1] W. Volknandt[1]; [1]Neurochemistry, Johann Wolfgang Goethe-University, Frankfurt, Germany, [2]Biochemistry I, Johann Wolfgang Goethe-University, Frankfurt, Germany, [3]Pharmaceutical Chemistry, Johann Wolfgang Goethe-University, Frankfurt, Germany

Synaptic transmission between neurons relies on synaptic vesicles. Therefore, the analysis of the synaptic vesicle proteome is essential for the identification of components involved in vesicle mobilization, migration to the presynaptic plasma membrane, docking and fusion as well as recycling of synaptic vesicles via endocytosis. To date, a considerable variety of synaptic vesicle proteins has been identified that govern these processes. To identify novel proteins and to complete the synaptic vesicle proteome, we subjected synaptic vesicles to different gel-based approaches in combination with mass spectrometry. Synaptic vesicles were isolated from rat brain synaptosomes by hypoosmotic shock and subcellular fractionation techniques as well as by immunoisolation. Proteins were separated using three different gel separation techniques and were analysed by mass spectrometry after tryptic in-gel digestion. Approaches comprised the two-dimensional gel systems BAC/SDS-PAGE and dSDS-PAGE along with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) as well as one-dimensional SDS-PAGE followed by nano-liquid chromatography/tandem mass spectrometry (nano-LC/MS/MS). We identified a total of ~230 proteins. In addition, we demonstrate that the three experimental systems supplement each other. 1D-electrophoresis lead to the identification of 180 proteins. For comparison we identified 94 and 93 proteins using BAC/SDS-PAGE and dSDS-PAGE, respectively. Only 42 proteins were identified in all three experimental approaches. Interestingly, a number of hitherto unknown proteins were identified, some of them by one approach, others by two. Taken together, these data demonstrate that the parallel application of different gel systems is essential for achieving a complete organelle proteome analysis.

1349

### *C elegans* UNC-44 Neural Ankyrin Binds to PP2A, CLASP2, and Calcium

P. Gong, A. J. Otsuka; Department of Biological Sciences, Illinois State University, Normal, IL

Neural UNC-44 AO13 ankyrin is required for proper axon guidance (Otsuka *et al., J. Cell. Biol*., 129:1081-1092, 1995; Boontrakulpoontawee and Otsuka, *Molec. Genet. Genom*., 267:291-302, 2002; Otsuka *et al*., *J. Neurobiol.*, 50:333-349, 2002). In yeast two-hybrids, the carboxyl domain of AO13 ankyrin interacted with members of two protein families: the protein phosphatase 2A (PP2A) B' subunits [encoded by *W08G11.4* and *C13G3.3* (Gong and Otsuka, *Molec. Biol. Cell* (suppl.), 14:124a, abst. #685, 2003)] and the +TIP, CLASP2, encoded by *cls-2*. By deletion analysis, W08G11.4 and C13G3.3a proteins interacted with a 141-aa region of UNC-44 (residues 6658-6798) containing a two-EF-hand domain. This region bound radioactive calcium in overlay assays. Site-directed mutations revealed that the EF hand 14-aa repeats [S(L/V)(T/S)SL(G/A)EFERLEKE] are important for W08G11.4 binding, but less so for C13G3.3a. Specifically, W08G11.4 binding is prevented by changes that alter the calcium-binding loops or charge clusters in the EF-hand stem. As expected for a S/T phosphatase substrate, phosphoserine mimics in the EF-hand loop regions did not prevent B'-subunit binding. The amino and carboxyl ends of W08G11.4 were found to be necessary for binding to UNC-44. There is the possibility that UNC-44 is a calcium sensor modulating, or modulated by, the UNC-44 phosphorylation state. Although CLASP2 associates

microtubules with the cell cortex, the adapter molecules remain unknown. In other systems, CLASP2 binds to the plus ends of microtubules at the leading edge of migrating cells, is inhibited by phosphorylation, and is controlled by PI3-kinase and GSK-3β pathways. In *C.* elegans, CLASP2 antibodies labeled the cytoplasm of many cells and, in some cases, labeled the cell periphery. The interaction between UNC-44 and CLASP2 leads to the hypothesis that UNC-44 may act to capture CLASP-tipped pioneer microtubules that are exploring the limits of growth cone filopodia.

1350

**An Investigation of the *C elegans* JIP3 Homolog, UNC-16, in Synaptic Vesicle Transport and Maturation**

H. M. Brown, A. Goncharov, D. Byrd, Y. Jin; Molecular, Cellular and Developmental Biology, University of California, Santa Cruz, Santa Cruz, CA

Synaptic vesicles are specialized carriers for neurotransmitters. Vesicular biogenesis at the Golgi and subsequent transport by microtubule-based motor proteins known as kinesins is essential for the accumulation of synaptic vesicles at synapses. Extensive biochemical, morphological and physiological studies, however, have shown that the chemical composition, the size and the shape of newly synthesized, actively transported synaptic vesicles differ from those that are present at functional synapses. These observations thus imply that synaptic vesicles require a "maturation" step to be accumulated at synapses and to carry out their function. At least two pathways are implicated in vesicle maturation; precursor vesicles may mature following an initial fusion with the plasma membrane or through an intermediate endosomal compartment. We have previously characterized the *C. elegans* JIP3 homolog, UNC-16, for its role in regulating synaptic vesicle localization. The JIP3 protein family has been shown to physically interact with the conventional kinesin-I complex, and function as cargo adaptors. Transport of synaptic vesicles also requires the UNC-104 kinesin. In further phenotypic and genetic double mutant analyses of *unc-16* and kinesins involved in synaptic vesicle transport, we have identified a kinesin-1 independent role for UNC-16. Cellular analysis of markers to multiple subcellular compartments shows that *unc-16* affects a specific subset of cargos, including endosomal compartment residents. Preliminary studies suggest that UNC-16 may be involved in synaptic vesicle maturation through endosomal regulators.

1351

**Fer Kinase Regulates Presynaptic Vesicle Localization Through β-catenin Regulation**

S. Lee,[1] S. Ng,[1] S. X. Bamji,[2] J. Balsamo,[3] J. Lilien,[3] L. F. Reichardt[1]; [1]Department of Physiology, University of California, San Francisco, San Francisco, CA, [2]Department of Cellular and Physiological Sciences, Brain Research Centre, University of British Columbia, Vancouver, BC, Canada, [3]Department of Biological Sciences, University of Iowa, Iowa City, IA

Cadherins are calcium-dependent intercellular adhesion molecules that have been proposed to play important roles in synaptic development in association with catenins and other signaling proteins. Recently β-catenin has been shown to function as a scaffold protein to recruit and anchor PDZ domain-containing proteins to cadherin clusters which in turn localize synaptic vesicles at nascent synapses. Maintenance of cadherin complexes is known to be regulated through phosphorylation and dephosphorylation of β-catenin, but the kinases and phosphatases that are responsible for β-catenin regulation during presynaptic development are not clear. Here we report the role of the nonreceptor tyrosine kinase Fer in presynaptic development. Inhibition of the association of Fer with p120 catenin by a cell-permeable peptide resulted in increased tyrosine phosphorylation of β-catenin and its dissociation from cadherin complexes in hippocampal neurons. Synaptic vesicle clusters were dispersed along the axons and dispersion was prevented by expression of a β-catenin mutant unable to be phosphorylated at a key cadherin interaction site (Y654F). The peptide also increased the dynamics of vesicle puncta movement significantly. Overexpression of a kinase-dead mutant of Fer also resulted in synaptic vesicle dispersion, implying that the kinase activity of Fer is required for proper localization of synaptic vesicles. Taken together, our results suggest that Fer, recruited by p120 catenin to the cadherin complex, may activate an adjacent phosphatases to dephosphorylate β-catenin, which enables β-catenin to act as a scaffold to localize synaptic vesicles.

1352

**Serum- and Glucocorticoid Inducible Kinase (SGK) Destabilizes Microtubules in Hippocampal Neurons**

Y. C. Yang,[1] C. H. Lin,[2] E. H. Y. Lee[1]; [1]Division of Neuroscience, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan Republic of China, [2]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan Republic of China

Tau is involved in the regulation of microtubule (MT) dynamics, axonal transport and neuronal morphology. Hyperphosphorylation of Tau proceeds to neurodegeneration in Alzheimer's disease. Previous reports have shown that mRNA of serum- and glucocorticoid inducible kinase (*sgk*) is increased at brain lesion site and after rats are exposed to an enriched environment. Enhanced expression of *sgk* was found to facilitate memory formation in rats. According to the AKT/SGK substrate motif on Tau, SGK might be a candidate Tau protein kinase. In the present study, we examined the role and mechanism of SGK-1 in regulation of Tau phosphorylation and MT polymerization. Results from kinase assay showed that active SGK (ΔSGK) specifically phosphorylated Tau at Ser214 both *in vitro* and *in vivo*. Immunostaining results indicated that SGK-1 is co-localized with Tau in hippocampal neurons. Further, SGK and phospho-Ser214 of Tau were found to co-localize and concentrate at where MT is disorganized. *In vitro* MT polymerization results revealed that ΔSGK disassembled MT through phosphorylation of Tau at Ser214. Transfection of pEGFP-*sgk* to cultured hippocampal neurons significantly inhibited neurite outgrowth. However, co-transfection of pEGFP-*sgk* and pDsRed-*tau*S214A reversed the impairing effect of pEGFP-*sgk* on hippocampal neurite outgrowth. Further, ΔSGK directly disassembled self-polymerized MT and taxol-induced pre-polymerized MT in a dose-dependent and ATP-independent manner. These results provide the first and direct evidence that SGK not only phsophorylates Tau to affect MT polymerization and neurite outgrowth, but also acts as a MT destabilizer to directly disassemble MT in hippocampal neurons. (This work was supported by a Grant from the National Science Council of Taiwan NSC 93-2321-B-001-009)

1353

**Recombinant Prion Protein Induces Rapid Polarization and Axonal Outgrowth in Embryonic Rat Hippocampal Neurons in Culture**

J. M. Kanaani (Kanaaneh),[1] S. B. Prusiner,[2] J. Diacovo,[1] G. Legname,[2] S. Baekkeskov[1]; [1]Dept. of Medicine, Microbiology/Immunology, and Diabetes Center, University of California San Francisco, San Francisco, CA, [2]Institute of Neurodegenerative Diseases, Dept. of Neurology and Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA

While a β-sheet-rich form of the prion protein ($PrP^{Sc}$) causes neurodegeneration, the biological activity of its precursor, the cellular prion protein ($PrP^{C}$), has been elusive. In this study, we have developed an *in vitro* assay to study the effect of purified recombinant prion protein (recPrP) on the polarization and neurite outgrowth of rat fetal hippocampal neurons in culture. Overnight exposure of one-day-old fetal rat hippocampal neurons for 22 h to Syrian hamster recPrP (SHaPrP) or mouse recPrP, folded into an α-helical rich conformation similar to that of $PrP^{C}$, dramatically enhanced the development of neuronal polarity and neurite extention in a dose-dependent manner. Thus, cultures treated with 0.45 μM SHaPrP showed a 1.9-fold increase in neurons with a differentiated axon, a 13.5-fold increase in neurons with differentiated dendrites, a five-fold increase in axon length, and the formation of extensive neuronal networks. Formation of synaptic-like contacts was increased by a factor of 4.6 after exposure to recPrP for seven days. Neither the N-terminal nor C-terminal domains of recPrP nor the PrP paralogue doppel (Dpl) enhanced the polarization of neurons. The pattern of immunostaining of neurons incubated with SHaPrP using antibodies specific for the hamster $PrP^{C}$, suggests binding of SHaPrP to a ligand that is distributed throughout the neuronal surface and that bound SHaPrP is not internalized. Inhibitors of PKC and of Src kinases, including p59Fyn, blocked the effect of recPrP on axon elongation, while inhibitors of phosphatidylinositol 3-kinase showed a partial inhibition, suggesting that signaling cascades involving these kinases are candidates for transduction of recPrP-mediated signals. The results predict that full-length $PrP^{C}$ functions as a growth factor involved in development of neuronal polarity.

1354

### The Receptor Tyrosine Kinase EphB2 is Polarized to the Dendritic Membrane of Cultured Hippocampal Neurons

K. Osborne,[1] I. Ethell[2]; [1]Neuroscience, University of California Riverside, Neuroscience Department, Riverside, CA, [2]Neuroscience, University of California Riverside, Biomedical Sciences Division, Riverside, CA

The receptor tyrosine kinase EphB2 has been shown to be important in the formation of dendritic spines in hippocampal neurons (Ethell et al., 2001; Penzes et al., 2003; Henkemeyer et al., 2003). The dendritic spines are postsynaptic sites of the majority of excitatory axon-dendritic synapses in the central nervous system, and their formation is a critical aspect of synaptic development. While there is increasing knowledge of Eph signaling, little is known about their localization. The differential targeting of Eph receptors may be important to their function in neurodevelopment, including cell migration, axon guidance and synaptogenesis. In synapses, it is traditionally thought that Ephs function postsynaptically while their ligands ephrins act presynaptically. Recent work has shown that the traditional synaptic arrangement of Ephs and ephrins can be inverted, as in the CA1 hippocampal region (Grunwald et al. 2004). Here we investigate the localization of EphB2 in hippocampal neuron cultures. In brief, we transfected cultures at 7 DIV with GFP and EphB2-Flag and evaluated the cell surface EphB2 axodendritc distribution at 8 DIV. Our preliminary data show that EphB2 is polarized to the somatodendritic domain of cultured hippocampal neurons. This is of particular importance since recent work has shown that dendritically localized EphB2 is needed for dendrite morphogenesis and maintenance of dendrites in mature neurons (Hoogenraad et al. 2005). Future work will consist of identifying sorting motifs governing the targeting of EphB2 to dendrites and examining the axodendritic distribution of EphB2 in CA1 and CA3 neurons as well as the role of EphB2 polarity in neuronal development. This work is supported by NIMH grant MH67121.

1355

### Brain-Derived Neurotrophic Factor Regulation of AMPA Receptor Trafficking

H. Nakata, S. Nakamura; Department of Biochemistry and Cellular Biology, National Institute of Neuroscience, Kodaira, Tokyo, Japan

The change of the number of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamatergic receptors (AMPARs) by neuronal activity is implicated in the synaptic plasticity. The activation of N-methyl-D-aspartate receptor (NMDAR) has been known to trigger the delivery of AMPARs into the synaptic membrane in hippocampal excitatory synapses. Brain-derived neurotrophic factor (BDNF) and other neurotrophins are also essentially involved in synaptic plasticity, at presynaptic and postsynaptic sites. Notably, BDNF was shown to unmask silent synapses in the developing mouse barrel cortex. Here, we investigated the role of BDNF on the AMPAR trafficking at postsynaptic sites of the cultured cortical pyramidal neurons. Using fluorescence resonance energy transfer (FRET) technology and Cameleon probe (A. Miyawaki, RIKEN) for $Ca^{2+}$ imaging, along with immunocytochemistry for the separate measurement of surface and intracellular AMPAR subunit GluR1, the dendritic spine-shaft pairs were assessed for the effect of BDNF and its signaling pathways involved. We report that BDNF application increased the surface expression of GluR1 at the postsynaptic densities, similar to the effect of NMDAR stimulation performed by two-photon uncaging of MNI-glutamate. The essential signaling was $Ca^{2+}$ transients evoked by the activation of receptor tyrosine kinase TrkB/PLCγ pathway. Both the intracellular $Ca^{2+}$ rise, $Ca^{2+}$ release from the intracellular $Ca^{2+}$ channel inositol trisphosphate receptor ($IP_3R$), and $Ca^{2+}$ influx through subsequent activation of store-operated cation channel TRPC (transient receptor potential canonical) contributed to the surface expression of GluR1 in the postsynapses. These results suggest an important role of BDNF in the regulation of AMPAR trafficking directly at the postsynaptic membrane via diverse $Ca^{2+}$ signaling.

1356

### Interaction of Neurotrophin-3 and Fibroblast Growth Factor (FGF2) in the Development of the Cochlear Nucleus (CN) of the Mouse Embryo

D. K. Morest; Neuroscience, University of Connecticut Health Center, Farmington, CT

How do NT3, FGF2, and their receptors affect cellular events in CN development? Immunohistochemistry was used to localize factors at particular stages of the normal morphogenetic sequence. An in vitro model was used to discover each factor's effects and interactions at particular stages in the sequence. By specific antibody staining in F1 offspring (CBA/JxC57BL/6J), TrkC protein appeared in the neural precursors prior to E11 and continued in the same cells until after birth. NT3 appeared in the precursor cells during migration (E13-E15) and disappeared shortly after birth (P1). TrkC and NT3 coincided in the same structures, including the formative phases of axons, terminals, and their synaptic targets. Thus NT3 tracks the morphogenetic sequence within a window defined by TrkC. In vitro, the anlage of the CN was explanted from E11 embryos. Neuroblasts migrated onto a Vitrogen substrate and began to differentiate, forming neurites, growth cones, and neuronal markers. The cultures were divided into seven groups and fed with defined medium, with or without FGF2, BDNF, and NT3 supplements, alone or in combinations, for 7 days. By time-lapse imaging, FGF2 alone had the most positive effect on proliferation and migration. FGF2 and NT3 produced the most increases, apparently synergistic, on neurite outgrowth and fasciculation. Synaptic vesicle protein (SV2) was more abundant in cultures treated with FGF2

and NT3 or BDNF. Immunocytochemical observations in vitro showed that NT3 and its receptor TrkC were greatest when cultures were treated with NT3 and FGF2 or BDNF. The results support the hypothesis that FGF2 promotes proliferation and migration, NT3 and BDNF stimulate neurite outgrowth, while NT3 and FGF2 promote fasciculation and synapse formation. Supported by NIH grant DC006387.

1357

**Dendritogenesis and Localization of NMDA and AMPA Receptors in Rat Primary Hippocampal Neurons are Controlled by an Endoplasmic Reticular Protein Sigma-1 Receptor**

S. Tsai, T. Hayashi, T. Su; Ddps/cnbr/irp, NIDA-NIH, Baltimore, MD

Sigma-1 receptors (Sig-1R) are endoplasmic reticular proteins that have been implicated in schizophrenia, Alzheimer's disease, depression, amnesia, and drug abuse via mechanisms that have yet to be totally understood. Sig-1R were shown by us to regulate the level of gangliosides which have been demonstrated at least to be important in dendritogenesis. We hypothesize therefore that one of the important functions of Sig-1R in the brain may involve dendritogenesis. We employed the small interfering RNA (siRNA) strategy to test this hypothesis in rat primary hippocampal neurons. A knockdown of Sig-R with the siRNA against Sig-1R causes a near depletion of gangliosides in the neuron. The siRNA treatment concomitantly impairs the growth and development of dendrites. Specifically, the siRNA-transfected neurons have shorter dendrites and possess fewer branches on the dendrite. The transfected neurons also form fewer dendritic spines while possessing more filopodia greater in length. These filopodia apparently fail to retract to form mature dendritic spines, thereby continuing to extend themselves as long filopodia. Further, the knockdown of Sig-1R by siRNA reduces the levels of synapse markers PSD-95 and synaptophysin, and dramatically changes the localization of NMDA receptors and AMPA receptors. NMDA receptors or AMPA receptors are present in the form of clusters in siRNA-transfected neurons, and exist mainly at the dendritic shafts and not at spine heads where they normally localize. These results suggest that Sig-1R play a critical role in dendritogenesis and in the maturation of dendritic spines perhaps by regulating the level of gangliosides in the brain. As gangliosides are important constituents of lipid rafts, it is tempting to speculate that Sig-1R may regulate dendritogenesis by affecting the formation of lipid rafts in the neuron.

1358

**Integrin-Dependent Remodeling of Dendritic Spines and Synapses in Cultured Hippocampal Neurons**

Y. Shi, I. M. Ethell; Division of Biomedical Sciences, Neuroscience Program, University of California, Riverside, Riverside, CA

The dendritic spines are postsynaptic sites of the majority of excitatory axon-dendritic synapses in the central nervous system and are responsible for synaptic transmission and synaptic plasticity. Many different factors and proteins have been shown to control dendritic spine development and remodeling. We and others have previously shown that interactions between the EphB family of receptor tyrosine kinases and their cognate ligands, the ephrin-Bs, play an important role in dendritic spine formation in hippocampal neurons. The extracellular matrix (ECM) components and their cell surface receptors, integrins, have been also found in the vicinity of synapses and shown to regulate synaptic efficacy and play an important role in long-term potentiation (LTP). The molecular mechanisms by which integrins affect synaptic efficacy have begun to emerge, however, their role in structural plasticity is poorly understood. Here we show that integrins are involved in dendritic spine/synapse remodeling in cultured hippocampal neurons. Beta 1, beta 3 and beta 5 subunits of integrin are localized in dendritic spines/synapses of hippocampal neurons in vitro and in vivo. Treatment of cultured hippocampal neurons with arginine-glycine-aspartate (RGD)-containing peptide, which mimicks effects of ECM on integrin signaling, induces elongation of existing dendritic spines and promoted formation of new filopodia. Moreover, these effects are also accompanied by F-actin reorganization and synapse remodeling. Furthermore, block of NMDA receptor by its antagonist MK801 prevents RGD-induced actin reorganization at synapses. Our data show that RGD treatment induces actin reorganization through NMDA receptor-mediated activation of calcium/ calmodulin-dependent kinase II (CaMKII) suggesting that integrin regulation of NMDAR/CaMKII-mediated actin reorganization might be responsible for ECM-mediated spine remodeling in hippocampal neurons.

1359

**Spin90 as a Novel Regulator of Dendritic Spine Morphology**

S. Lee, K. Lee, S. H. Kim, W. K. Song, S. Chang; Department of Life Science, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

SPIN90 (SH3 Protein Interacting with Nck, 90 kDa) is an Nck-binding protein that contains one SH3 domain, three PRDs and a serine/threonine-rich region. SPIN90 has been known to regulate actin polymerization and is highly expressed in the brain, but its role in the neuronal system is unknown. We have found that in addition to its localization in presynaptic terminals, SPIN90 highly exists in the dendritic spines of cultured hippocampal neurons. It turned out that localization of SPIN90 in the dendritic spines is due to its association with PSD-95 through PRD-SH3 interaction. To further investigate the role of SPIN90 in the dendritic spines, we overexpressed SPIN90 in hippocampal neurons in culture. When expressed in early developing neurons, SPIN90 induced a dramatic increase in dendritic filopodial length and number. Consistently, upon expressing it in mature neurons, the protrusions emerging from dendritic shaft of transfected neurons show significant increase in average length and density. The knockdown of expression by SPIN90 specific siRNA dramatically reduces dendritic spine density. Finally we proved that chemically induced LTP has resulted in the clustering of SPIN90 into dendritic spines. Our results suggest that SPIN90 associates with PSD-95, thus localizing in the dendritic spines where it modulates actin dynamics to regulate dendritic morphology. Our results also raise the possibility that SPIN90 could be the regulator of dendritic spine morphology during the synaptic plasticity. Table of Contents

## Golgi Complex (1360-1382)

1360

**Subcellular Mechanisms in Monocrotaline-induced Pulmonary Hypertension: Megalocytosis, Golgi Blockade and Enhanced Unfolded Protein Response (UPR)**

S. Mukhopadhyay,[1] P. B. Sehgal[2]; [1]Cell Biology and Anatomy, New York Medical College, Valhalla, NY, [2]Cell Biology and Anatomy and Medicine, New York Medical College, Valhalla, NY

Monocrotaline-induced pulmonary hypertension in the male rat is an irreversible hit-and-run disease process in that a single exposure to this plant alkaloid [half-life of active pyrrolic derivative (MCTP) is ~3 sec in water] leads to clinical manifestations (pulmonary hypertension) 10-14 days later. The cellular consequences include enlargement of endothelial and smooth muscle elements in pulmonary arteries as well of Type II alveolar epithelial cells ("megalocytosis"). These enlarged "growing but non-dividing" cells contain increased ER, enlarged Golgi and exhibit reduced cell-cycle traverse. We have previously proposed that MCTP leads to a block in ER-Golgi trafficking and alters the mitosis-sensor functions of the Golgi (the Golgi blockade hypothesis) resulting in disruption of caveolin-1/raft function and a block of entry into mitosis. We now report that there is an activation of the UPR sensor pathways in MCTP-induced megalocytosis. In confocal immunofluorescence assays of megalocytotic lung epithelial cells (A549), Ire1α was translocated to the nucleus, while ATF6 and XBP-1 accumulated in a tight perinuclear region together with the Golgi marker GM130. PERK showed strong perinuclear and nuclear accumulation and there was in increase its target phospho-eIF2α, which also showed marked nuclear accumulation. Additionally, the chaperone ERp57/Grp58 showed nuclear translocation, while protein disulfide isomerase (PDI) gained a strong circumnuclear provenance. By Western Blotting, there was an increase in the cellular levels of the transcription factors STAT3, STAT1 and XBP-1 as well as an increase in nuclear phospho-eIF2α. These data indicate that the UPR pathways are activated following MCTP-induced Golgi Blockade and this likely contributes to the development of the megalocytosis phenotype.

1361

**Morphological Alterations of Cellular Secretory System by Human Cytomegalovirus**

J. Seo,[1] W. J. Britt[2]; [1]Microbiology, School of Medicine, University of Alabama at Birmingham, Birmingham, AL, [2]Microbiology, Pediatrics, Neurobiology, School of Medicine, University of Alabama at Birmingham, Birmingham, AL

The function of cellular organelles in the assembly of large DNA viruses such as human cytomegalovirus (HCMV) is not well understood. Studies of herpesvirus assembly have identified a membranous, juxtanuclear compartment, the virus assembly compartment (AC), as a site of virion structural protein accumulation late in infection. We hypothesized that cells infected with HCMV undergo morphological changes of compartments within the secretory system that promote formation of the AC. We initiated studies to define the role of viral functions in the formation of this modified cellular compartment. Late in infection, components of the secretory system appeared to be organized into concentric shells that surrounded viral proteins present in the interior of the AC. Protein markers of the ERGIC initially were widely distributed in the infected cell but coalesced late in infection into a more discrete structure that remained peripheral to the AC. Markers of the TGN were found in the inner layers of this structure late in infection. Interestingly, the distribution of the TGN was more diffuse early in infection and overlapped with the juxtanuclear structure, but late in infection it was localized to the outer layers of the AC. The adapator protein AP-1 overlapped with TGN, while AP-3 co-localized with the viral proteins in the AC late in infection. Because the juxtanuclear structure appeared to be an immature, less well defined structure early in infection that compacted as the infection progressed, it appears that morphoplogic alterations in the cellular secretory pathway are linked to virus assembly and that these changes correlate temporally with virus production. Our findings suggest that the morphological alteration of cellular secretory system induced during viral assembly in HCMV infected cells could represent virus-specified modification of cellular organelles to facilitate cytoplasmic assembly of this DNA virus.

1362

**pH-dependent Golgi Retention of β4-Galactosyltransferase I**

B. E. Schaub, E. G. Berger, J. Rohrer; Institute of Physiology, University of Zurich, Zurich, Switzerland

β-1,4-galactosyl- (GalT) and α-2,6-sialyltransferase (SiaT) co-localize in the trans cisternae of the Golgi apparatus (GA) where they catalyze elongation and termination of complex N-glycan chains, respectively. Upon addition of monensin, SiaT remains in the GA whereas GalT dissociates into swollen vesicles, where it co-localizes with trans-Golgi network (TGN) markers such as TGN46 and cation-independent mannose-6-phosphate receptor. Both GalT and SiaT contain four domains, the cytoplasmic tail (CT), the transmembrane domain (TMD), the stem region (STEM), and the catalytic domain. To determine the domains involved in the monensin response of GalT, we created several chimeric constructs where we swapped domains of GalT with those of SiaT and replaced the catalytic domain with GFP. Furthermore, we also mutated the potential phosphorylation sites in the CT of GalT. These constructs were stably transfected in HepG2 cells to study the dynamics of response to monensin by time-lapse video microscopy. The localization patterns of the GalT-SiaT-GFP chimeras were compared to endogenous giantin by means of double immunofluorescence of fixed cells and as time lapse immunofluorescence videos in living cells, using a Laser Scanning Confocal Microscope. Our results strongly suggest that the CT is necessary for monensin sensitivity of GalT whereas the TMD and the STEM regions might play a supporting role. Moreover preliminary data show that phosphorylation of GalT is not required for monensin sensitivity. Supported by SNF 31-66578.01 to EGB and SNF 31-67274.01 to JR.

1363

**Discovery of the Molecular Determinants of GlcNAc6ST-1 Function**

M. M. Desko, J. J. Kohler; Chemistry, Stanford University, Stanford, CA

The Golgi-resident enzyme *N*-acetylglucosamine-6-sulfotransferase-1 (GlcNAc6ST-1) is responsible for the sulfation of GlcNAc on the L-selectin ligand residing on endothelial cells, which is implicated in lymphocyte homing. GlcNAc6ST-1 is a type II transmembrane protein consisting of a cytosolic N-terminal domain, a transmembrane domain, a proline-rich stem region that is 75 amino acids in length and a catalytic domain; the stem region and catalytic domain both extend into the Golgi lumen. Flow cytometry data obtained using the L-selectin-ligand specific antibody G72 show that mutation of proline residues within the stem region affects the ability of GlcNAc6ST-1 to sulfate GlcNAc in the context of the tetrasaccharide sialyl Lewis$^x$. The proline to alanine mutation also affects the subcellular localization of the protein. Conversely, mutation of stem region asparagine and threonine residues has no effect on enzyme localization or enzyme activity. GlcNAc6ST-1 has been previously reported to form a homodimer. To study dimerization of GlcNAcST-1, we developed a new microscopy assay. Our assay relies on the ability of the C-terminal KDEL sequence to direct localization to the endoplasmic reticulum (ER). HeLa cells transfected with EYFP-tagged GlcNAc6ST-1 alone show normal Golgi localization of the enzyme, as analyzed by confocal microscopy. When HeLa cells are transfected with both EYFP-tagged GlcNAc6ST-1 and KDEL-tagged GlcNAc6ST-1, EYFP fluorescence is observed in the ER, indicative of the formation of higher order enzyme complexes. Our assay provides an efficient way to determine the molecular determinants of GlcNAcST-1 dimerization and will also be applicable

to other Golgi-resident enzymes, several of which have been shown to form homo- and heterodimers.

1364

**Rab3D Localizes to Early Golgi Compartments in Rat Intestinal Goblet Cells**

L. van Weeren,[1] A. M. de Graaff,[1] A. Ultee,[2] J. A. Valentijn[3]; [1]Biochemistry and Cell Biology, University of Utrecht, Faculty of Veterinary Medicine, Utrecht, The Netherlands, [2]Pathobiology, University of Utrecht, Faculty of Veterinary Medicine, Utrecht, The Netherlands, [3]Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

The small GTP-binding protein, rab3D, has been implicated in regulated exocytosis for its predominant localization to secretory granules in various cell types. However, deletion-mutation studies have suggested that rab3D is rather involved in the biogenesis of secretory granules. In support of this, it has been shown in several cell types that rab3D also localizes to the trans-Golgi network (TGN). We here report the surprising observation that rab3D associates with early Golgi compartments in intestinal goblet cells and in Brunner's gland acinar cells. Rab3D expression in intestine was demonstrated by SDS-PAGE and Western blotting of homogenates prepared from rat duodenum and colon. In addition, rab3D was detected in lysates of human colonic mucous epithelial cells (HT29-5M21). Confocal laser-scanning microscopy revealed rab3D immunofluorescence in goblet cells of duodenum and colon. Rab3D was also present in duodenal Paneth cells and Brunner's gland acinar cells. In these two cell-types rab3D localized to secretory granules while in the latter, the Golgi area also displayed strong labeling. In the goblet cells of both duodenum and colon, rab3D labeling was concentrated in the Golgi area whereas mucous granules were almost devoid of signal. In colon, rab3D immunofluorescence from goblet cells was stronger in the crypts than in the villi. There was no co-localization between rab3D and the TGN marker, TGN-38. In contrast, rab3D co-localized partially with the cis-Golgi marker, GM-130, and with the cis- to trans-Golgi marker, beta-COP. Strong co-localization was observed between rab3D and the lectins, *Griffonia simplicifolia* agglutinin II, and soybean agglutinin, which have been described as markers of medial- and cis-Golgi, respectively. Post-embedding immunoelectron microscopy confirmed the rab3D localization to cis- and medial-Golgi cisternae. Taken together, these data suggest a novel role for rab3D in an early Golgi trafficking pathway.

1365

**A Member of the Dynein Light Chain Roadblock/LC7 Family, Robl1, Interacts Differentially With Rab6A and Rab6A'**

B. Wanschers,[1] R. van de Vorstenbosch,[1] S. M. King,[2] J. Fransen[1]; [1]Cell Biology, NCMLS, Nijmegen, The Netherlands, [2]University of Connecticut Health Center, Farmington, CT

Small GTPases of the Rab family are key regulators of intracellular membrane transport. To date more then 60 members, including isoforms, are recognized. In the past we have identified three Rab6 isoforms: Rab6A, Rab6A' (generated via alternative splicing of a duplicated but distinct exon within the Rab6A gene) and Rab6B (a brain specific isoform). Rab6A is implicated in the regulation of retrograde intra-Golgi and Golgi to ER transport whereas Rab6A' in addition can regulate a transport step from late endosomes to the Golgi apparatus, characterized by the internalization of Shiga toxin (Mallard *et al*., J Cell Biol 156, 653-64, 2002). In a search for specific Rab6 interacting proteins, several novel binding partners were identified. One novel interactor is Robl1, a member of the dynein light chain family that is structurally related to proteins which have been implicated in modulating the GTPase activity of small GTPases (Koonin and Aravind, Curr Biol 10, R774-6, 2000). The interaction between Rab6 isoforms and Robl1 was further established by co-immunoprecipitation studies. Interestingly, pull-down assays with either GTPγS- or GDP-loaded Rab6A or Rab6A' revealed that Robl1 preferentially binds to Rab6A when it is GTP-bound and to Rab6A' in its GDP-bound state. Microscopic studies demonstrated that Robl1 colocalizes with Rab6 at the Golgi apparatus and is actually recruited there by Rab6. Given the nature of the Robl1 protein, we also checked whether expression of Robl1 has an effect on the intrinsic GTPase activity of Rab6 isoforms. However we did not observe such a modulating effect. On the basis of these data we suggest that the Robl1-Rab6 interaction may act as an anchor for attaching vesicle- or Golgi membranes to the dynein complex.

1366

**Trs130 is Required for the Specificity Switch of A Dual-Ypt/Rab GEF**

N. Segev, N. Morozova, Y. Liang, R. Cox, S. Chen, E. Yoo; Biological Sciences, University of Illinois, Chicago, IL

Intracellular trafficking in eukaryotes is regulated by Ypt/Rab GTPases. These GTPases are activated by guanine-nucleotide exchange factors (GEFs). When in the GTP-bound state, Ypt/Rabs interact with effectors that mediate individual steps of the protein trafficking pathways. An attractive possibility is that Ypt/Rabs and their accessory factors not only regulate the separate steps of protein transport pathways, but also coordinate these separate steps. Here, we propose a novel mechanism for such coordination in the Golgi. The modular multi-protein Golgi-associated complex, TRAPP, acts as a GEF for Ypt1 and Ypt31/32, the GTPases that regulate entry into and exit from the yeast Golgi, respectively. Trs130 is a TRAPP subunit that is found in late but not early Golgi, is conserved from yeast to man, and is a candidate for several human disorders. We show that Trs130 determines the specificity of the dual-GEF complex: it is required for switching the GEF activity of TRAPP from a Ypt1-GEF to a Ypt31/32-GEF. Unlike the wild-type complex, TRAPP purified from trs130ts mutant cells neither contains the Trs130 protein, nor acts as a Ypt31 GEF. However, its Ypt1-GEF activity is higher than that of the wild type. We propose that TRAPP acts as a Ypt1 GEF in early Golgi, whereas in late Golgi Trs130 is required for switching this activity off, and turning the Ypt31/32 GEF activity on. Such a switch-able dual-specificity GEF would ensure activation of Golgi Ypts at the correct cisterna, thereby coordinating entry into and exit from the Golgi apparatus.

1367

**Mammalian GRIP-domain Proteins Form alpha-helical Homodimers Which Are Localised to Distinct Sub-domains of the trans-Golgi Network**

F. J. Houghton, M. R. Luke, M. A. Perugini, P. A. Gleeson; Biochemistry & Molecular Biology, University of Melbourne, Bio21, Parkville, Australia

Intracellular transport from the TGN (trans-Golgi network) to the plasma membrane has been found to involve a family of golgin proteins, all of which contain the GRIP or 45-residue Golgi targeting domain (1,2). On the basis of the high content of heptad repeats, GRIP domain proteins are predicted to contain extensive coiled-coil regions that have the potential to mediate protein-protein interactions. Four mammalian GRIP domain proteins have been identified which are targeted to the TGN through their GRIP domains: p230, golgin-97, GCC88 and GCC185 (Golgi localised

coiled-coil protein). Here we have investigated the ability of the four mammalian GRIP domain proteins to interact. Using a combination of immunoprecipitation experiments of epitope-tagged GRIP domain proteins, cross-linking experiments and yeast two-hybrid interactions, we have established that the GRIP proteins can self-associate to form homodimers exclusively. Two-hybrid analysis indicated that the N- and C- terminal fragments of GCC88 can interact with themselves, but not with each other, suggesting that the GRIP domain proteins form parallel coiled-coil dimers. Analysis of purified recombinant golgin-97 by CD spectroscopy indicated a 67% alpha-helical structure, consistent with a high content of coiled-coil sequences. These results support a model for GRIP domain proteins as extended rod-like homodimeric molecules. The formation of homodimers, but not heterodimers, indicates that each of the four mammalian TGN golgins has the potential to function independently. This is consistent with recent data, which indicates that these golgins are localised to distinct sub-domains of the TGN (4,5). 1. Brown et al. (2001). Traffic 2,:336-344, 2. Luke et al (2003). J Biol Chem 278, 4216-4226, 3. Gleeson et al (2004). Traffic 5,: 315-326. 4. Luke et al (2005). Biochem J 388, 835-841 5. Lock et al (2005). Submitted

1368

**Trans-Golgi network Golgins**

M. C. Derby, P. A. Gleeson; Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, Melbourne, Australia

The four mammalian golgins, p230/golgin-245, golgin-97, GCC88 and GCC185, are targeted to trans-Golgi network (TGN) membranes[1] where they are proposed to play a role in the maintenance of TGN structure or protein trafficking. All four proteins exist as homodimers, and are predicted to form extensive regions of coiled-coils. They share a modestly conserved C-terminal region, the GRIP domain, which is responsible for their G-protein-dependent recruitment to the TGN. Membrane recruitment of both p230 and golgin-97 is mediated by ARF-like GTPase 1 (Arl1)[3]. Using transient transfections of cultured mammalian cells, confocal microscopy and microRNA interference (miRNA) we explored the TGN localization and function of GCC88 and GCC185. When expressed as tagged versions in the same cell, the GRIP domains of GCC88 and GCC185 are both localized to the TGN, but display only partial overlap. Overexpression of full-length GCC185 resulted in the loss of GCC185 Golgi staining, and the appearance of small GCC185-labelled punctate structures. These GCC185-labelled structures did not affect the TGN localization of the other GRIP proteins. Unlike p230 and golgin-97, GCC88 and GCC185 do not interact with Arl1 in vivo, indicating that the GRIP domains differ in their specificity for G proteins. Depletion of GCC185 protein levels by miRNA results in a disruption of GM130 and p230, from tight Golgi staining to extended or large punctuate staining, implicating GCC185 in the maintenance of Golgi structure. These differences in localization and binding at the TGN indicate that the mammalian GRIP proteins may be recruited by different mechanisms to distinct sub-domains of the TGN. We propose that GCC185, along with the other GRIP domain proteins, functions to maintain the integrity of distinct TGN domains. 1. Luke, ML *et al* (2003) *J. Biol. Chem.* **278** 4216-26 2. Lu, L *at al* (2003) *Mol. Biol. Cell* **14** 3767-81

1369

**Characterization of the Golgi Matrix Proteins Grh1p and Uso1p in Pichia pastoris**

S. K. Levi; Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

The Golgi plays a central role in the secretory pathway, and it consists of membranous cisternae that sort newly made proteins to various intra- and extracellular destinations. In most species, the Golgi cisternae are stacked in an ordered fashion. A set of proteins that collectively form the Golgi "matrix" has been implicated in generating this stacked structure. The matrix is also thought to play a role in vesicle tethering to the Golgi, and has recently been shown to interact with signaling proteins, suggesting that it may serve as a structural scaffold for the Golgi. However, there are conflicting data that suggest different mechanisms by which the matrix functions, and the identities of the matrix components are not known with certainty. I am characterizing two putative Golgi matrix proteins in the budding yeast *Pichia pastoris*. Unlike *Saccharomyces cerevisiae*, *P. pastoris* has a stacked Golgi. Grh1p, a homolog of mammalian GRASP65, co-localizes with the transitional endoplasmic reticulum marker Sec13p. Grh1p exhibits dynamic behavior typical of Golgi resident proteins. Uso1p, a homolog of the mammalian Golgins p115 and GM130, also localizes in the vicinity of the Golgi. Analysis of the Uso1p domains that display homology to GM130 and to p115 is currently underway. Our preliminary results indicate that *P. pastoris* is a powerful model system for analyzing the Golgi matrix.

1370

**Characterization of the Golgin Proteins Rud3 and Coy1 in *Pichia pastoris***

L. A. Satkamp,[1] B. S. Glick[2]; [1]Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, [2]Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

A substantial fraction of proteins produced in eukaryotic cells traverse the Golgi apparatus, an organelle responsible for targeting and modification of cellular proteins. Typically, a Golgi consists of stacks of pancake-like cisternae that represent biochemically distinct cis, medial and trans compartments. The molecular interactions governing the stacking of these cisternae are thought to involve so-called golgin proteins. Golgins are coiled-coil proteins believed to be necessary for vesicle tethering and for the structure of Golgi stacks. These proteins contain specific domains for association with Golgi membranes and with particular proteins, which include other golgins as well as certain GTPases. Evidence is increasing for regulatory functions of golgins, in addition to their structural roles. For example, a role for the golgin GRASP65 has been established in the regulation of spindle dynamics in mammalian cells. Homologues to mammalian golgins have been identified in the budding yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*. Surprisingly, a stacked structure is not necessary for secretion in budding yeasts. The Golgi cisternae of *S. cerevisiae* rarely form stacks and are dispersed throughout the cytosol, while the cisternae of *P. pastoris* do form discrete stacks of 3-5 cisternae. A comparative analysis of golgins in these two systems provides a unique opportunity to identify specific proteins and domains important for Golgi stacking. The goal of this project is to investigate the role of two golgins, Rud3 and Coy1, in the stacking of Golgi cisternae in *P. pastoris*. Initial experiments will involve a basic characterization of the functions and localizations of these proteins by microscopy. Subsequent studies will explore how the specific properties of Rud3 and Coy1 may contribute to the distinct types of Golgi organization in the two yeasts.

1371

**Golgins in the Golgi of *Trypanosoma brucei***

I. Barinaga-Rementeria Ramirez,[1] A. Price,[2] H. Ho,[1] C. He,[1] J. Yelinek,[1] G. Warren[1]; [1]Cell Biology, Yale University School of Medicine, New Haven, CT, [2]VaxInnate, New Haven, CT

The protozoan parasite, *T. Brucei*, is proving to be a useful organism to study the biogenesis of the Golgi apparatus. There is only a single Golgi stack which duplicates by a process of de novo biogenesis, with components being supplied to the new Golgi by both the old Golgi and the new ER exit site. Golgins are coiled-coil proteins that have been implicated in the structure and function of the Golgi apparatus. To study their role during biogenesis there is the need to identify *T. Brucei* homologs. This process has been confounded by the difficulty of distinguishing golgins from other coiled-coil proteins in the *T. Brucei* database. To overcome this hurdle we have been exploiting the fact that many mammalian golgins are Rab effectors. The *T. Brucei* homolog of Rab1 has been identified and expressed as a GST-construct. An enriched Golgi fraction has been purified from the parasite using a putative GlcNAc transferase as the marker. Incubation of detergent extracts of these Golgi membranes with immobilized Tb-Rab1 has led to the identification of several candidate proteins.

1372

**Role of Basic Amino Acid Residues in the Cytosolic Region of Type II Membrane Proteins on Golgi Localization in Plant Cells**

K. Matsuoka, S. Takata, K. Toyooka, Y. Goto; RIKEN Plant Science Center, Yokohama, Japan

Using membrane anchored prolyl hydroxylase (PH) as a model protein, we have been analyzing the transport of type II membrane proteins from the endoplasmic reticulum (ER) to the Golgi apparatus in plant cells. We reasoned the role of basic amino acid residues in the cytosolic region of PH using a transient expression system and GFP fusion technique. We found that two basic amino acids joined together or separated with one or two amino acids was sufficient for the efficient Golgi localization. Using this information, and proteome information of Arabidopsis we predicted possible type II membrane proteins that localize to the Golgi apparatus and subsequent organelles. We found that many type II homologs for glycosyl transferases and several classes of plant specific proteins contain this localization signal before their type II sinal anchor. Currently we are analyzing the localization of some of these proteins. Results of these analyses and related analyses on the role of COPII proteins for the ER export will be discussed.

1373

**The COG Complex-mediated Recycling of Golgi Enzymes is Essential for Normal Protein Glycosylation**

A. Shestakova, S. Zolov, V. Lupashin; Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR

The Conserved Oligomeric Golgi (COG) complex is the cis-Golgi localized oligomer responsible for intra-Golgi transport and glycoprotein modification. The COG complex consists of eight subunits, Cog 1-4 (Lobe A) and Cog5-8 (Lobe B) and is proposed to be involved in the tethering of recycling intra-Golgi vesicles to appropriate Golgi cisternae. In this work we used prolonged siRNA-induced knockdown of Cog3p (COG3 KD) in Hela cells to uncover the molecular link between function of the COG complex and defects in protein glycosylation. Immunoanalysis of prolonged COG3 KD cells showed enhanced degradation of Golgi enzymes, resulting in a defective glycosylation of plasma membrane (CD44) and lysosomal (Lamp2) glycoproteins. Although immunoblotting after acute COG3 KD did not detect any change in SDS-PAGE mobility of glycoproteins, pulse chase technique revealed heterogeneity in CD44 and Lamp2 glycosylation, arguing that defective glycosylation arises soon after the COG complex dysfunction and is being exaggerated with duration of COG3 KD. Localization of underglycosylated CD44 and Lamp2 was similar in control and COG3 KD cells, indicating that the COG complex was not directly required for anterograde traffick and protein sorting in mammalian cells. For the first time we demonstrated that in COG3 KD cells components of Golgi glycosylation machinery were redistributed to COG complex dependent (CCD) vesicles. These vesicles were analyzed by glycerol velocity gradient and *in vitro* system, which reconstitutes vesicle docking/fusion. COG7 KD caused similar defects in respect to both Golgi trafficking and protein glycosylation. We conclude that the COG complex functions to tether constantly recycling transport vesicles carrying Golgi enzymes, thus providing appropriate localization of Golgi glycosylation machinery.

1374

**Biogenesis of the Golgi Apparatus in *Trypanosoma Brucei*: Digital Reconstructions from Serial Thin Section Electron Micrographs**

J. T. Yelinek, C. Y. He, H. H. Ho, G. B. Warren; Department of Cell Biology, Yale University, New Haven, CT

The biogenesis of the Golgi apparatus is comprised of two phases: duplication and partitioning. A variety of protozoa have been used to study the duplication phase of the Golgi biogenetic pathway due to their simple morphology and the presence of a single Golgi stack.. One such organism is *T. brucei*, the causative agent of African sleeping sickness in man and Nagana in ruminants. *T. brucei* is also well suited for morphological studies due to its small size, simple anatomy, and ordered duplication and division of organelles. These traits allow complete cells to be imaged both at the level of light and electron microscopy, and placed in the context of the cell cycle based on morphological markers of development. Photobleaching experiments using *T. brucei* have shown that the old Golgi contributes material to the growing new Golgi. Fluorescence microscopy also shows that a new ER export site appears concomitantly with the new Golgi. The ultrastructural morphology of Golgi intermediates, as well as the earliest stages of Golgi growth, cannot be identified at the level of light microscopy. This requires electron microscopy coupled with peroxidase cytochemistry of HRP-tagged Golgi proteins. By combining this with serial sectioning and 3D rendering, we hope to provide a complete picture of the Golgi during the cell cycle of this protozoan parasite.

1375

**MEK1 and the Importance of Golgi Unlinking for Mitotic Entry**

T. N. Feinstein, M. A. Puthenveedu, A. D. Linstedt; Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

The role of the protein kinase MEK1 at the G2/M cell cycle transition remains controversial. Inhibition of MEK1 by RNA interference or by using the MEK1/2-specific inhibitor U0126 caused a significant delay in the passage of synchronized HeLa cells into M-phase. Consistent with the hypothesis that the delay was caused by a block in a MEK1 pathway acting to disperse the Golgi apparatus, the MEK1 dependency at G2/M was abrogated if Golgi proteins were dispersed prior to M-phase by treatment of cells with brefeldin A. MEK1 may act on the Golgi to unlink the

membranes or to cause their repositioning. As a test we depleted GRASP65 which we found unlinks Golgi stacks without altering their positioning. Strikingly, cells with unlinked Golgi stacks due to GRASP65 knockdown were MEK1-independent at G2/M suggesting that the MEK1 pathway is required to unlink the Golgi for normal mitotic entry. To test for a direct role, we expressed a nonphosphorylatable form of the GRASP family member GRASP55, which we had previously shown undergoes MEK1-dependent mitotic phosphorylation. Expression of the GRASP55-S222,225A, but not wildtype, blocked progression into mitosis. These findings support a model in which unlinking of the Golgi ribbon by MEK1-dependent GRASP phosphorylation is a prerequisite for normal mitotic entry, possibly by releasing and/or activating pro-mitotic signaling factors.

1376

**Correlated Light and Electron Microscopy of Golgi During Cell Division**

G. M. Gaietta,[1] B. N. G. Giepmans,[1,2] T. J. Deerinck,[1] J. Llopis,[3] L. Ngan,[1] S. R. Adams,[2] R. Y. Tsien,[2,4] M. H. Ellisman[1]; [1]Neurosciences / NCMIR, UC San Diego, La Jolla, CA, [2]Pharmacology, UC San Diego, La Jolla, CA, [3]Facultad de Medicina y C.R.I.B., Universidad de Castilla - La Mancha, Albacete, Spain, [4]Howard Hughes Medical Institute, UC San Diego, La Jolla, CA

The fate of the Golgi apparatus during mitosis is controversial, with evidence for (Lippincott-Schwartz & Zaal, 2000) and against (Warren, 1993; Yaffe, 1996; Nunnari & Walter, 1996, Pecot & Malhotra 2004) coalescence with the endoplasmic reticulum. We recently developed methods that facilitate correlated light microscopic studies of living cells followed by electron microscopic examination using the same molecular labeling system (Gaietta et al., 2002). We have now generated and imaged a fusion consisting of the first 117 residues of the mouse isoform of alpha-mannosidase II (containing the cytoplasmic, transmembrane and part of the luminal domains), GFP, a linker ESSGS, and finally the newly optimized tetracysteine motif FLNCCPGCCMEPGGR (Martin et al, 2005). When expressed in HeLa cells, this chimeric protein specifically localizes in the trans-Golgi network (TGN). The mannosidase component guarantees docking into the Golgi membrane, with the fluorescent protein / tetracysteine tag within the lumen. The GFP is visible at light microscopic resolution without further treatment, whereas the tetracysteine tag can be acutely reduced with a membrane-permeant phosphine, labeled with ReAsH, monitored in the light microscope (especially 2-photon excitation), and photoconverted for electron microscopy, allowing correlated 4-dimensional optical recording on live specimens followed by ultrastructural analysis of the same regions by electron tomography. We find in HeLa cells that at cytokinesis the Golgi generally forms four co-linear clusters, two per daughter cell. Within each daughter, the smaller cluster near the midbody gradually migrates to rejoin the major cluster on the far side of the reforming nucleus. The vesicles remain in close association with the microtubular network and generally remain distinct from the endoplasmic reticulum during mitosis. Supported by NIH-RR04050 and NIH-1P20-GM72033.

1377

**PKA Inhibition Triggers Golgi Autophagy**

E. Bejarano, M. Cabrera, L. Vega, J. Hidalgo, A. Velasco; Cell Biology, University of Seville, Seville, Spain

Protein kinase A (PKA) is stably associated to the Golgi complex during interphase. To analyze the role of PKA in Golgi structural maintenance cells were depleted of PKA RIIα regulatory subunits using a specific interfering RNA (siRNA). Under these conditions, PKA Cα catalytic subunits redistributed to the cytosol and the Golgi complex underwent fragmentation. By electron microscopy Golgi fragments were found to correspond to vesicular structures reminiscent of autophagosomes. Indeed, Golgi disruption induced by siRNA incubation was abrogated by treatment with the autophagy inhibitor 3-methyladenine. Furthermore, Golgi fragments colocalized with LC3, a marker of the autophagosome membrane. A similar effect was originated by inducing PKA displacement from the Golgi complex to cytosol indicating that the disassembly process was due to PKA mislocalization. Inhibition of PKA catalytic activity also gave rise to enzyme redistribution and autophagic Golgi disassembly. In contrast, PKA activation protected the Golgi complex from disruption during the autophagic response developed by cells subjected to amino acid starvation. These data suggest a role for Golgi-associated PKA activity in autophagy regulation. They also indicate that part of the autophagy process involves dismantling and degradation of the Golgi complex

1378

**ER-Derived Assembly of a Functional Golgi Apparatus in HeLa Cells Reflects the Recycling Kinetics of Golgi Membrane Proteins**

S. Jiang, B. Storrie; Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR

In yeast, Golgi apparatus assembly is from ER-exit sites in a manner consistent with the predictions of cisternal maturation, i.e., cis-first assembly. We have tested whether this outcome is true for ER-derived Golgi apparatus assembly in HeLa cells. We find that brefeldin A (BFA) + H89 treatment substantially disperses all Golgi proteins into an ER-like distribution. Golgi glycosyltransferases, e.g., co-distribute with ER membranes by co-staining with the ER markers, PDI or Sec61p. Golgi matrix protein distribution is slightly granular and co-localizes with residual ER-exit site staining. Upon drug washout, all four Golgi matrix proteins accumulated with the same kinetics into post-ER, nascent Golgi structures. The matrix binding transmembrane protein, giantin, exhibited the same rapid accumulation. Significantly, other Golgi transmembrane proteins such as Golgi glycosyltransferases, glycosidases, TGN46 and GPP130 accumulated in newly formed Golgi structures more slowly and importantly in the same time order as their recycling kinetics as revealed by an ER-exit block or overexpression of Rab6a. Kinetics was trans-first. Restricting nascent Golgi structures to close proximity to ER-exit sites by microtubule depolymerization accelerated Golgi assembly. With a 2 h 15°C-block in the presence or absence of nocodazole, however, Golgi glycosyltranferase accumulation remained in the ER. Golgi functionality in cargo transport was a late event and required full Golgi assembly. The comparative kinetics of ER-derived Golgi assembly was the same with BFA treatment alone. We propose a model of ER-derived Golgi assembly in mammalian cells in which assembly is nucleated by matrix proteins, follows a trans-first order, and is related to the ER-cycling kinetics of Golgi proteins. Supported by a grant from the National Science Foundation.

1379

**Golgi Fragmentation During Apoptosis : Does the Cytoskeleton Play a Role?**

S. Mukherjee, R. Chiu, L. Novikov, D. Shields; Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY

The Golgi apparatus exists as a series of membrane stacks arranged in a perinuclear lace-like reticulum whose structure is maintained, in part, by the actin and tubulin cytoskeleton. In mammalian cells the Golgi apparatus undergoes extensive fragmentation during apoptosis. Several laboratories including our own have shown that a number of coiled-coil proteins (golgins) involved in maintaining Golgi structure and function,

including p115 (~962 residues) are cleaved during apoptosis. p115 is cleaved by caspases-3 and -8 to generate a C-terminal fragment (CTF) of ~205 residues, which when expressed in otherwise healthy cells potently induces apoptosis**.** By using deletion analysis, we have identified a 25 amino acid motif, whose expression is sufficient to cause apoptosis in a variety of cell types. To further investigate the mechanism of Golgi fragmentation during apoptosis, we have now analyzed the role of the cytoskeleton. When apoptosis was induced by treating cells with Fas antibody to activate the Fas receptor or with staurosporine, a general kinase inhibitor, Golgi fragmentation and cleavage of some golgin polypeptides preceded changes in microtubule organization. Additionally, no significant changes in microtubule morphology were evident early during the apoptotic response, although the Golgi apparatus was fragmented completely. In contrast, the organization of the actin cytoskeleton was differentially sensitive to apoptosis inducing agents. During staurosporine treatment, dramatic changes in actin morphology preceded Golgi breakdown, whereas in Fas treated cells, actin fibers were relatively unaffected. Furthermore, while the Golgi apparatus was fragmented completely during apoptopsis, little change was observed in ER organization. Our results suggest that during the induction of apoptosis, disruption of the actin or tubulin cytoskeleton is not a necessary prerequisite for Golgi fragmentation. Thus Golgi breakdown during apoptosis is a consequence of caspase mediated cleavage of several Golgi matrix proteins.

1380

**Centrosome and Golgi Complex Reorganization during Myogenesis Can Proceed without a Coherent, Dynamic Microtubule Network**

K. J. M. Zaal,[1] E. Reid,[1] G. G. Gundersen,[2] T. Zhang,[1] E. Ralston[1]; [1]NIAMS, NIH, Bethesda, MD, [2]Anatomy/ Cell Biology, Columbia University, New York, NY

During differentiation of skeletal muscle cells, microtubule-organizing sites (MTOC) are redistributed to the nuclear membrane and to cytoplasmic sites. The Golgi complex (GC) is fragmented and its elements, associated with ER exit sites (ERES), redistribute to the nuclear membrane as well. It is assumed that microtubule reorganization causes GC reorganization. To test this, we have assessed the reorganization of GC, MTOC, and ERES in C2 muscle cells with disrupted or massively stabilized microtubule networks. Myoblasts cultured with 200 ng/ml nocodazole in both growth and differentiation media rounded up, failed to fuse, and retained only small fragments of microtubules. Nevertheless, differentiated (myogenin-positive) cells showed a normal MTOC redistribution. GC achieved a circumnuclear redistribution in 70% of the cells, but a “perfect” redistribution in only 10%. Myoblasts overexpressing a C-terminal fragment of EB1 lost directed motility and formed abnormally wide, short myotubes with a disrupted microtubule network, but showed normal MTOC and GC redistribution. Treatment with 10 to 250 nM taxol stopped cell motility and affected cytokinesis but not MTOC reorganization. GC reorganization took place but was often incomplete. ERES often failed to redistribute, even in cells in which the GC did. Finally, treatment with the ruthenium complex 1-OH, a specific GSK3-beta inhibitor (Williams et al, 2005, Angew. Chemie, 44, 1984) reduced microtubule dynamics without preventing MTOC reorganization. However, GC was bipolar rather than circumnuclear in 70% of these cells. Thus, microtubule manipulation allowed us to uncouple MTOC, GC, and ERES reorganizations and to show that 1) MTOC reorganization is resistant to alterations of the microtubule network; 2) it is a prerequisite of GC reorganization, while ERES reorganization is not; 3) GC reorganization is initiated but only rarely completed in the absence of normal microtubules.

1381

**Reassembly of an Intact Golgi Complex Requires Both Phospholipase $A_2$ ($PLA_2$) Activity and Microtubules**

B. L. Judson, W. J. Brown; Molecular Biology and Genetics, Cornell University, Ithaca, NY

We have previously found that $PLA_2$ antagonists cause the normally interconnected Golgi ribbon to break up into large fragments of stacked Golgi cisternae that remain located in the juxtanuclear region. Using the reversible $PLA_2$ antagonist, ONO-RS-082, it appeared from static immunofluorescence images that following washout of the drug, disconnected stacks rapidly and dramatically sprouted membrane tubules that served to reassemble an intact Golgi ribbon. Considering these results together, we concluded that the maintenance and assembly of normal Golgi architecture is dependent on the $PLA_2$-mediated, dynamic formation of inter-Golgi membrane tubules. Here we have used live-cell, time-lapse microscopy to image the reformation of Golgi complex during the reassembly process. Using HeLa cells stably expressing GFP-galactosyltransferase, we found that fragmented Golgi ribbons undergo a burst of tubule formation following washout of ONO-RS-082. Quantifying these results shows an obvious difference: $4.3 \pm 3.8$ tubules/cell/10 min before washout; $29.9 \pm 11.9$ tubules/cell/10 min after washout. These tubules often formed bridges between separate stacks, although tubules also extended out into the cytoplasm. Whether or not there was directed movement of tubules from one stack to another could not be determined. However, we have found that formation of these inter-stack tubules was facilitated by microtubules because treatment with nocodazole greatly inhibited their formation and reassembly into an intact Golgi ribbon. These studies show that a late stage of Golgi reassembly occurs via membrane tubules, whose formation is dependent on $PLA_2$ activity and microtubules. (Supported by NIH grant DK51596)

1382

**Identification of a Lysophospholipid Acyltransferase with a Novel Role in Golgi Membrane Dynamics**

J. A. Schmidt, W. J. Brown; Molec. Biol. and Genetics, Cornell University, Ithaca, NY

Phospholipase ($PLA_2$) and lysophospholipid acyltransferase (LPAT) activities appear to have antagonistic roles in the formation of Golgi membrane tubules and retrograde trafficking to the ER: Golgi tubule formation is promoted by $PLA_2$ activity and inhibited by LPAT activity. These enzymes may alter the shape of membrane bilayers to regulate the formation of trafficking intermediates. The overall goal is to identify specific LPATs that regulate Golgi membrane dynamics. The DNA sequences for six putative human transmembrane (TM) LPATs have recently been discovered; however, the functions of most are unknown. Here we report the identification of a specific TM LPAT (LPATγ) that regulates Golgi membrane tubule formation. When transfected into HeLa cells, LPATγ-GFP was primarily localized to the Golgi complex and also to ER membranes. Interestingly, over-expression of LPATγ significantly inhibited the rapid formation of Golgi membrane tubules that are induced by brefeldin A (BFA). Additionally, retrograde trafficking of Golgi membranes to the ER in BFA-treated cells was significantly slower. LPATγ may control Golgi membrane dynamics either by aiding in the fission of Golgi-derived vesicles that consume membrane tubules, or by re-acylating tubule-forming lysophospholipids that were generated by $PLA_2$s. These data are the first to show a direct role for a TM LPAT in regulating Golgi membrane trafficking. (Supported by NIH grant DK51596)

## Epithelia II (1383-1396)

1383

### Three Color Image Collection for Segmentation and Analysis of Protein Subcellular Location Patterns in Polarized Monolayers of CaCo2 Cells

E. M. Goellner,[1] E. Garcia Osuna,[2] R. F. Murphy[3]; [1]Chemical Engineering and Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, [2]Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, [3]Biological Sciences and Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA

Automated analysis of the location of proteins is useful in determining statistical differences in protein patterns that may be undetectable to the human eye. The goal of this project is to collect three-dimensional, three-channel fluorescence microscope images of the CaCo2 cell line. CaCo2 are human colon cancer epithelial cells, which will be used as a model system for extending current automated subcellular location pattern analysis methods to polarized epithelia and tissues. Unlike in prior experiments on single cultured cells, the polarized monolayers require a method for determining the boundaries of each cell before pattern analysis can be accomplished. Achieving an acceptable method of data collection to facilitate segmentation has been a main goal of this project. The protocol we have developed involves tagging a protein of interest such as a Golgi or mitochondrial protein using primary antibodies and Alexa-488 conjugated secondary antibodies. In order to perform automated segmentation, it is necessary to also have a nuclear image and a continuous plasma membrane image. DRAQ5 was used to stain the nuclei. A primary antibody against the cadherin family of proteins and a secondary antibody conjugated to Alexa-564 dye provided the continuous plasma membrane staining desired. This staining was homogeneous and easily recognized by automated segmentation algorithms. The resulting three-color images are appropriate for automated segmentation as well as subcellular location pattern analysis of the proteins of interest. Future work will include results for automated classification of proteins imaged using this protocol.

1384

### Immunohistochemical Study on the Distribution of Sodium Dependent Vitamic C Transporters in the Adult Rat Brain

D. H. Shin, G. H. Mun, M. J. Kim, J. H. Lee, J. Y. Park, J. S. Kang, J. H. Kim, Y. I. Hwang, H. J. Kim, Y. B. Chung, S. H. Oh, W. J. Lee; Department of Anatomy, Seoul National University College of Medicine, Seoul, Republic of Korea

Sodium-dependent Vitamin C co-transporters (SVCTs) was known to transport the reduced form of ascorbic acid into the cell. And they were reported to be expressed in different types of the cells within the various organs. Especially in central nervous system, only *SVCT2* mRNA was known to be expressed mainly in neurons and some types of neuroglial cells. However, since there were very few data on the expression of SVCT proteins in the brain, we tried to report the comprehensive data on the distribution of SVCT proteins in the rat brain using immunohistochemical technique for the first time. In our study on the adult rat brain, SVCT2 IRs were intensely localized in the neurons of cerebral cortex, hippocampus, and Purkinje cells of cerebellum while much weaker SVCT2 IRs were also found in the other brain regions as well. Judging from double immunohistochemical data, most of the cells expressing SVCT2 IRs were likely to be neurons or microglia even though the cells in choroids plexus or ependymal cells around the ventricles also exhibited SVCT2 IRs. As the subcellular localization of SVCT proteins is necessary for understanding the exact role of the protein, the current overall mapping of SVCT IRs in the rat brain could be the basis for the further studies on the relating subject. *Shin DH and Mun GH contributed equally to this study. **For correspondence, please send E-mail to kinglee@snu.ac.kr

1385

### Differential Expression of Zinc Transporters between Fetal (FHs) and Adult (Caco-2) Human Intestinal Cells Results in Divergent Effects of Zinc Exposure

M. Jou,[1] S. L. Kelleher,[1] A. Philipps,[2] B. Lonnerdal[1]; [1]Nutrition, University of California Davis, Davis, CA, [2]Pediatrics, University of California Davis Medical School, Davis, CA

Zinc (Zn) homeostasis in adults is primarily regulated by changes in intestine expression and localization of Zn-specific transporters such as Zip4 (primary Zn importer), ZnT-1 (Zn efflux), ZnT-2 (vesicular Zn sequestration) and MT-1 (intracellular metal-chelator). However, Zn absorption and excretion is higher and less regulated, respectively, in premature infants, suggesting that mechanisms regulating intestinal Zn homeostasis may be immature. We used cultured fetal (FHs) and adult (Caco-2) intestine cell lines to determine differences in Zn uptake, Zn transporter expression (MT-1, ZnT-1, ZnT-2 and Zip4) and effects of acute (1h) Zn exposure (50µM). While basal Zn uptake rates were similar between cell lines, Zn uptake was higher (3-fold) in fetal cells which were also more sensitive to Zn exposure than adult cells (FHs, LD50 = 75µM; Caco-2, LD50 =135µM). Our data suggest that these differences may be a result of differential expression of Zn-specific transporters as fetal cells do not express Zip4, but have higher Zip1 (2-fold) and Zip3 (3-fold) and lower ZnT-1 (~50%) and ZnT-2 (~20%) expression compared to adult cells. While acute Zn exposure post-transcriptionally increased Zip4 and ZnT-1 protein levels resulting in no effect on MT-1 (an indicator of cellular stress or Zn levels) in adult cells, Zn exposure increased MT-1, ZnT-1 and ZnT-2 mRNA and ZnT-1 protein levels in fetal cells. We speculate this difference is due to the inability of fetal cells to tightly regulate Zn uptake as a result of the absence of Zip4 expression. In conclusion, our data suggests that the lack of Zip4 expression in fetal intestine cells may be responsible for differences in Zn absorption observed between preterm infants and adults, resulting in the lack of sensitive regulation at this age.

1386

### Mammary Gland Zinc Transport is Regulated by Prolactin through Changes in Zip3 and ZnT2

S. L. Kelleher, W. Chowanadisai, B. Lonnerdal; Nutrition, University of California Davis, Davis, CA

The mammary gland transports an extraordinary amount of zinc (Zn) to meet neonatal requirements. Human milk normally contains adequate Zn to meet these requirements; however, numerous reports have documented transient neonatal Zn deficiency in breast-fed infants as a consequence of low milk Zn concentration which cannot be corrected by maternal Zn supplementation. We have previously identified several Zn transporters in the mammary gland; however, mechanisms which regulate Zn transport into milk are not well understood. To examine this regulation we used gene silencing to knockdown Zip3 and ZnT2 in mouse mammary epithelial cells (MECs) which resulted in reduced Zn uptake (25% of normal) and Zn efflux (50% of normal), respectively, indicating that Zip3 and ZnT2 play major roles in mediating Zn uptake and secretion into milk. To verify the

dependence of human MEC Zn secretion on ZnT2, we identified a woman with low milk Zn concentration (0.3 mg Zn/L) and documented a point mutation in exon 2 of SLC30A2 (ZnT2) that results in a substitution of arg for his at amino acid 54. Using site-directed mutagenesis we determined that this mutation results in mis-localization of ZnT2 from the Golgi to a peri-nuclear compartment in MECs, likely preventing Zn efflux through the secretory pathway. Prolactin treatment (1μM) of normal mouse MECs resulted in Zip3 accumulation at the plasma membrane concurrent with increased Zn uptake as well as increased Zn secretion across the luminal membrane concurrent with increased ZnT2 protein abundance and ZnT2 movement to a vesicular compartment. Taken together, our results indicate that Zip3 and ZnT2 play major roles in mammary epithelial cell Zn uptake and efflux, respectively and suggest that prolactin plays a regulatory role in mediating Zn transport into milk.

1387

## GPR4, a Proton-Sensing Receptor in the Kidney

X. Sun, S. Petrovic; Medicine, University of Cincinnati, Cincinnati, OH

A G-protein-coupled receptor, GPR4, functions as a proton-sensing receptor stimulating cAMP production. pH-dependent stimulation of cAMP production occurs within the physiological pH range. Interestingly, GPR4 is highly expressed in the lungs and kidney, the two organs essential for blood pH regulation. However, the cellular distribution of GPR4 in these tissues is currently unknown. This study was designed to determine the localization of GPR4 in the mouse and rat kidney. By using real-time PCR, we find that GPR4 mRNA is expressed in the kidney cortex and medulla at the similar level. Immunofluorescent labeling of the kidney tissue sections with GPR4 antibody reveals GPR4 expression in the nephron segments resembling kidney collecting ducts. We used aquaporin 2 (AQP2) antibody as a marker of the kidney collecting duct. The doublelabeling with GPR4 and AQP2 antibodies demonstrates the co-localization of GPR4 and AQP2 in the kidney collecting duct. The collecting duct is comprised of two types of polarized epithelial cells: intercalated cells (ICs), critical for the acid-base regulation, and principal cells, which handle salt and water homeostasis and selectively label with AQP2 antibodies. The doublelabeling studies indicate that GPR4 is expressed in both cell types either uniformly distributed throughout the cytoplasm, or confined to the apical membrane and subapical vesicles. In some cells, labeling is present in the basolateral membrane. We also examined rats subjected to the increased acid or alkaline load. GPR4 immunostaining is much more pronounced in the kidneys of acid-loaded then alkali-loaded animals. In conclusion, the proton-sensing receptor GPR4 is selectively expressed in the kidney collecting duct, the nephron segment critical for the final adjustment of acid-base status. A change in acid-base status affects GPR4 expression. This data indicate that GPR4 may act as an acid-sensor in the kidney.

1388

## Intracellular $Ca^{2+}$ Changes of Vomeronasal Organ; Imaging Analysis of Response to Pheromones

H. Akutsu, Y. Satoh; School of Medicine, Iwate Medical University, Morioka, Iwate, Japan

[Objective] Sensory neuron of vomeronasal organ (VNO) may detect pheromones in various materials including urine. In present study, we investigated whether VNO of male rats respond to female urine. To this end we observed changes of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), which play a key role in cell functions. Imaging for $[Ca^{2+}]_i$ changes in intact tissues can provide a overview of physiological responses, therefore we prepared VNO specimens keeping intrinsic structural integrity. [Materials & Methods] VNO was dissected from adult male Wistar rat (10-14 weeks, body weight: 230-300g) and slice specimens (150 μm in thick) were cut by a vibratome. After the $Ca^{2+}$-sensitive dye loading, the slice specimens were set on cover-glass of perfusion chamber. The changes of $[Ca^{2+}]_i$ of VMO were imaged and analyzed by a real-time confocal laser scanning microscope (Nicon RCM/Ab). To avoid photodamage, images were acquired at 1 sec interval. Urine was collected from female Wistar rat (8-14 weeks, diestrous and proestrous), and diluted 1:1000 with HEPES-buffered Ringer solution (HR). [Results] Few spontaneous oscillatory fluctuations of $[Ca^{2+}]_i$ were observed. During perfusion of diluted urine from proestrous females, some epithelial neurons showed transient increase of $[Ca^{2+}]_i$. Oscillatory fluctuations were also observed. After the urine perfusion, the oscillatory response occurred in other neurons, although the urine was washout by normal HR. The response diminished when extracellular $Ca^{2+}$ was removed. Response to male or diestrous female urines were less. [Conclusion] Imaging of $[Ca^{2+}]_i$ changes on tissue specimens indicated that the heterogeneous responses of neurons of VNO. Various substances in estrous female urine induced responses in different manner, for example; in some cases, remove of urine substances induces cell response. The imaging method is useful to evaluate pheromone mechanism.

1389

## Model Wounds to Study the Collective Motility of Epithelial Cells

M. Poujade,[1] E. Grasland-Mongrain,[1] B. Ladoux,[2] A. Buguin,[1] P. Silberzan[1]; [1]Section de Recherche, Institut Curie, Paris, France, [2]MSC, Université Paris VII, Paris, France

The mechanical wounding of an epithelial monolayer triggers the motility of the border cells who then migrate to heal the monolayer while keeping intact the cell-to-cell contacts. However, the exact mechanism for the acquisition of this collective motility remains unclear. Some models involve the death of the cells that are removed from the wound. Some others are based on a permeabilization of the border cells. The signaling between cells through autocrine growth factor production is also often cited. In the experiments presented here, the wound does not damage the cells. We perform this task by using microfabricated structures to mask part of the surface to the cells during the growth of the film. To this end, we make thin masks out of a silicone elastomer by micro-molding. When the cell monolayer reaches confluence, the mask is removed without damaging the cells and we monitor the healing of this model wound by time lapse video microscopy. By using microfabrication, we can test many wounds whose widths vary from 20 μm to 500 μm or more, in a parallel way. We find that the presentation of free surface to a monolayer of MDCK cells in sufficient to trigger the motile phenotype. The cells invade this surface at a rate comparable to previous studies using damaging wounds. We have precisely measured this velocity of healing and find that the position of the border is a parabolic function of time ($X \alpha t^2$). This behavior is markedly different from the spontaneous filling of naturally occurring holes within a monolayer when it approaches confluence. In this last case, the motility is driven by the cell proliferation whereas it is only of secondary influence in the wound healing situation.

1390

## ERK1/2-dependent Phosphorylation and Relocalization of HSP27 in Keratinocytes Undergoing DLK-induced Differentiation

H. Robitaille,[1] R. Blouin,[2] K. Robitaille,[2] L. Germain[1]; [1]Laboratoire de Recherche des Grand Brûlés/LOEX and Département de Chirurgie,

Université Laval, Québec, PQ, Canada, [2]Département de Biologie, Université de Sherbrooke, Sherbrooke, PQ, Canada
Hsp27 is part of the small heat shock proteins (sHsp) family. It is involved in many cellular processes including cell death and differentiation. Indeed, Hsp27 is an important anti-apoptotic protein but also a molecular chaperone protecting the cell from protein agregation of misfolded proteins. In t27 is associated with keratin filaments in the very late phase of keratinocyte differentiation. We previously showed that overexpression of DLK, a MAPKKKinase preferrentially expressed in the granular layer of the epidermis, induces the terminal differentiation of keratinocytes and activate the ERK1/2 and JNK MAPK pathways. In this study, we studied the expression and subcellular distribution of Hsp27 during epidermal cell differentiation using both an in vitro model of reconstructed skin and cultures of normal keratinocytes infected with an adenoviral vector for DLK. The MEK inhibitor PD98059 and the JNK inhibitor SP600125 were also used to determine the role of these pathways in the distribution and the phosphorylation of Hsp27 during keratinocyte differentiation. We first showed that Hsp27 is upregulated during keratinocyte differentiation (in vivo and in vitro) and is also phosphorylated on serine 82 in the very late phases of keratinocyte differentiation. Moreover, our results indicate that Hsp27 became insoluble and phosphorylated in normal human keratinocytes 48 hours after expression of DLK. The insolubility and the phosphorylation of Hsp27 in DLK-expressing keratinocytes were partially inhibited by the MEK inhibitor PD98059. Taken together, these results suggest that DLK may regulate the subcellular distribution and phosphorylation of Hsp27 through a mechanism involving ERK1/2 activation during human keratinocyte differentiation.

1391

**Rapid Inhibition of STAT1 Signalling in Epithelia by TGFβ**

C. Reardon, D. M. McKay; Intestinal Disease Research Program, McMaster University, Hamilton, ON, Canada
Background: Interferon-γ (IFNγ) and transforming growth factor-β (TGFβ) are multi-functional cytokines that are key regulators of mucosal immunity, typically functioning in a reciprocal cross-inhibitory manner. Objective: To determine the ability of TGFβ to modulate IFNγ-induced signal transducer and activation of transcription-1 (STAT1) signalling in enteric epithelial and immune cells. Methods: Epithelia (Hep-2, HT-29) or mononuclear cells (THP-1) were pre-treated with TGFβ (1ng/mL; 0-60min), exposed to IFNγ (20ng/mL; 30min) and STAT1 DNA-binding activity, phosphorylation, and methylation was assessed by electrophoretic mobility shift assays (EMSA), immunoblotting, and autoradiography. Some epithelia were transfected with an expression plasmid encoding, SMAD7 which blocks TGFβ-SMAD signalling. Results: Epithelia, but not mononuclear cells, pre-treated with TGFβ for 15-60 min were hypo-responsive to IFNγ stimulation as indicated by reduced STAT1 DNA binding on EMSA (n=3-4). Despite this, immunoblotting for tyrosine$^{701}$ or serine$^{727}$-STAT1 revealed no difference in IFNγ ± TGFβ treated cells (n=3), indicating that the effects of TGFβ are downstream of IFNγ receptor-Janus kinase-STAT1 phosphorylation events. Additionally, STAT1 methylation was unaltered in IFNγ ± TGFβ treated cells (n=4). Functionally, IFNγ-induced upregulation of interferon response factor 1 (IRF-1; a STAT1 dependant gene) in epithelia was blocked by TGFβ pre-treatment. The ability of TGFβ to interfere with IFNγ-STAT1 signalling was not observed in epithelia over-expressing SMAD7. Conclusions: These results indicate that TGFβ activated SMAD signalling rapidly suppresses IFNγ-driven STAT1 signalling by reducing DNA-binding without effecting STAT activation; an event seemingly specific to epithelia. We speculate that this is a novel mode of action of TGFβ, and that full delineation of this mechanism may be a novel way to block the effects of pro-inflammatory cytokines and enhance the effect of TGFβ's reparatory properties.

1392

**Skin Barrier Acidity Measurement of Young and Aged Mice Using Two-photon FLIM**

P. Xu, E. Choi, M. Dermerjian, D. Crumrine, S. Chang, T. Mauro; Dermatology, UC San Francisco, San Francisco, CA
OBJECTIVE: It has been understood that one important factor related to the upper epidermis (stratum corneum) barrier function, is the epidermal acidity and pH gradient. We investigated in this paper the difference in acidity between young and aged mice, and for each case, its acidity in intracellular and extracellular domains. METHODS: Two-photon fluorescence lifetime imaging (FLIM) is used to identify pH values within the stratum corneum. The fluorophore, 2',7'-bis-(2-carboxyethyl)-5/6-carboxyfluorescein (BCECF), has a lifetime dependent on the ambient pH. Hairless mice, aged 12weeks and 12months, are used as models for young and aged skin. RESULTS: All measurements revealed acidic to neutral trend among the intra- and extra- cellular pH in both young and aged, with exception that the aged mice has a nearly flat intracellular acidity. It is also consistent that in both cases, extracellular domain is more acidic than intracellular by less than one unit of pH, while they converge towards the end of the SC at around 10 um deep. Young mice exhibit a more acidic SC barrier than the aged counterpart by 1-2 units of pH. The relative value between the intra- and extra- cellular pH is more random in the aged mice than the young ones. CONCLUSIONS: The hypothesis that young skin has a more acidic barrier than aged was proved experimentally and quantitatively. The overall trend from acidic to neutral along the depth of the SC is identical from the young and the aged, but the detailed gradient is different. Physiological mechanisms causing the difference, and pathological difference manifested by the difference, are topics to be studied further.

1393

**Keratin Filament Organization Defines Distinct Populations of Epithelial Stem Cells Within the Bulge of Vibrissae Hair Follicles**

D. Larouche,[1] X. Tong,[2] C. J. Roberge,[1] P. A. Coulombe,[2] L. Germain[1]; [1]Department of Surgery, Laboratoire d'Organogenèse Expérimentale (LOEX), Hôpital du Saint-Sacrement, Université Laval, Québec, PQ, Canada, [2]Departments of Biological Chemistry and Dermatology, The Johns Hopkins University School of Medecine, Baltimore, MD
Defining the intrinsinc features of post-natal stem cells (SC) is of great interest given their relevance to tissue homeostasis and therapeutic applications. The bulge, a specialized region of the outer root sheath (ORS) in hair follicles, houses a large fraction of stem cells in hair-covered skin. Focusing on rodent vibrissae follicles, which feature a prominent bulge, we used light and electron microscopy along with keratin expression to typify candidate stem cells exhibiting slow-cycling properties. Two types of slow-cycling cells occur in the vibrissae bulge. One group, Bb cells, occurs in the outermost basal layer of the bulge and exhibits a loosely organized keratin network correlating with expression of K5, K15, K17 and K19. The other group, Bs1 cells, occurs in the suprabasal layers proximal to Bb cells and feature a very prominent network of densely bundled keratin filaments correlating with K5/K17 expression. These filament bundles are missing in K17 null mice, giving rise to cell fragility within the Bs1 compartment, which likely accounts for the absence of vibrissae hair shafts in these mice. Bulge epithelial cells located in the more internal suprabasal layers (Bs2 cells) are contiguous with ORS from the lower portion of the follicle. Bs2 cells are not slow-cycling and contain K5, K6,

K14, K15, and K17, which presumably yields a keratin network of intermediate density. These findings significantly extend recent studies pointing to heterogeneity within the hair follicle stem cell niche, identify specific keratins as effectors of this heterogeneity, and point to a special role for K5/K17 filaments in a newly defined subset of candidate stem cells. Supported by the CIHR and the NIH. LG is holder of the Canadian Research Chair (CIHR) on Stem Cells and tissue engineering

1394

**Toll-like Receptors are Expressed in Cholangiocytes and Mediate Epithelial Defense Response to Cryptosporidium parvum Infection via Upregulation of Beta-defensin 2**

S. P. O'Hara, X. Chen, J. B. Nelson, P. L. Splinter, N. F. LaRusso; Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, Rochester, MN

Infection of epithelial cells by *Cryptosporidium parvum* triggers a variety of host cell innate and adaptive immune responses including release of cytokines/chemokines and upregulation of anti-microbial peptides. The mechanisms that trigger these host-cell responses are unclear. Thus, we evaluated the role of Toll-like receptors (TLRs) in host-cell responses during *C. parvum* infection of cultured human biliary epithelia (i.e., cholangiocytes). We found that normal human cholangiocytes in situ and in culture express all known TLRs. Moreover, *C. parvum* infection of cultured human cholangiocytes induced the recruitment of TLR2 and 4, but not other TLRs, to the infection sites. Activation of downstream effectors of TLRs including interleukin-1 receptor-associated kinase (IRAK), p-38, and NF-kB, was detected in infected cholangiocytes. Transfection of cholangiocytes with dominant negative (DN) mutants of TLR2 and 4 and myeloid differentiation protein 88 (MyD88), an adaptor protein for TLRs, inhibited the activation of IRAK, p-38 and NF-kB induced by *C. parvum*. Moreover, *C. parvum* upregulated the message and protein of human ß-defensin (HBD) -2, but not HBD-1 and HBD-3, in directly infected cells; also, inhibition of TLR2, 4 and MyD88 with DN mutants were each associated with a reduction of *C. parvum*-induced HBD-2 expression. A significantly higher number of parasites were detected in cells transfected with MyD88-DN than in control cells at 48-96 hours after exposure to parasites, suggesting MyD88-deficient cells were more susceptible to infection likely due to repression of HBD-2 expression. Consistent with this interpretation, we found that addition of recombinant HBD-2 to sporozoite preparations significantly decreased parasite viability. These findings demonstrate that cholangiocytes express a variety of TLRs, and suggest that TLR2 and TLR4 mediate cholangiocyte defense responses to *C. parvum* via upregulation of HBD-2.

1395

**Characterizing a Mutation that Prevents Pharyngeal Morphogenesis in *C elegans***

V. Praitis,[1] M. Miller,[1] A. Schacht,[1] J. Mushi,[1] Z. Yilma,[1] L. Yohannes,[1] S. Kniss[2]; [1]Biology, Grinnell College, Grinnell, IA, [2]Biology, University of Chicago, Chicago, IL

In *C. elegans*, pharyngeal morphogenesis is divided into several discreet steps including the creation of a pharyngeal primordium, reorientation of anterior pharyngeal cells, formation of stable epithelium between the buccal cells, pharynx, and intestine, and changes in cell shape (Portereiko & Mango, 2001). We have identified a weakly semi-dominant temperature-sensitive embryonic lethal mutation that causes defects in pharyngeal morphogenesis, resulting in embryos with the *pun* (*p*harynx *un*attached) phenotype. *ru5* embryos grown at the restrictive temperature undergo normal embryonic development until the bean stage when pharynx and mouth epithelial sheets fail to connect to each other. Temperature shift experiments show the altered gene product is only required during pharyngeal morphogenesis. To further characterize the *ru5* phenotype, we examined the expression pattern of proteins found in the pharynx and head hypodermis during this stage of development. Our results indicate that early developmental events and the establishment of the pharyngeal primordium occur normally in *ru5* embryos grown at the restrictive temperature. Reorientation of the anterior pharynx also appears normal, as the expression patterns of anterior markers such as AJM-1 and SMA-1 in *ru5* embryos are indistinguishable from wild type. However, the localization of these proteins in the arcade cells and in the head hypodermis is abnormal and the invagination associated with the buccal cavity does not occur in *ru5* embryos. These results suggest the failure to form a stable epithelium is due to defects in the organization, movement, or shape of the arcade cells or anterior hypodermis. We are currently working to identify the *ru5* gene product.

1396

**Characterization of the SH3BP4 C-Terminal Death Domain**

E. D. Koppelman, J. B. Kolberg, J. R. Dunlevy; Anatomy & Cell Biology, University of North Dakota, Grand Forks, ND

SH3BP4 has multiple protein-protein interaction domains at the N-terminus that are characteristic of the Eps15-Homology (EH) network family of proteins involved in endocytosis and intracellular sorting. SH3BP4 also contains a C-terminal death domain (DD) characteristic of proteins involved in apoptosis. Currently, this combination of protein-protein binding domains has not been reported in other proteins. The objective of this study is to characterize the subcellular localization and morphological effects of SH3BP4 death domain over-expression. Cultured human retinal pigment epithelial (ARPE-19) cells were transiently transfected with N-terminal Binding Domain (BD), full-length and C-terminal Death Domain (DD) SH3BP4 fusion proteins for 24, 48 and 72 hours. Western blot analysis was used to determine fusion protein levels while microscopy studies were used to characterize subcellular localization and nuclear morphology. Fusion protein levels were found to be elevated at 24 and 48 hours, but decreased substantially by 72 hours for each SH3BP4 construct while subcellular localization studies showed a distinct localization pattern for each SH3BP4 fusion protein. The N-terminal BD protein showed diffuse cytoplasmic and nuclear localization. The full-length fusion protein localized primarily to the plasma membrane and nuclear periphery. The C-terminal DD localized primarily to the plasma membrane and these cells also exhibited a rounded morphology. In nuclear morphology assays, an increase in nuclear condensation was observed for each of the SH3BP4 constructs compared to non-expressing controls. However, cells expressing the C-terminal DD had an increase in the percentage of cells with condensed nuclei compared to the SH3BP4 N-terminal BD and full-length fusion proteins. In conclusion, this study showed that the C-terminus of SH3BP4 may be critical for plasma membrane localization and that over-expression of the C-terminal DD appears to induce nuclear condensation and cell rounding which are morphological characteristics of apoptosis.

## Cancer II (1397-1417)

1397

### Structure and Functions of the Cytoplasmic Body Formed by Adenovirus E1B 55-kDa Protein

D. Liao, L. Y. Zhao, J. Liu; Anatomy and Cell Biology, University of Florida, Gainesville, FL

The adenovirus E1B 55-kDa protein (E1B) binds to N-terminal domain of p53 (TAD) and suppresses p53 activities. E1B forms a cytoplasmic body that retains p53, and this sequestration correlates with its ability to inhibit p53-mediated apoptosis. Mutagenesis revealed that p53 residues 23-27 are strictly required for p53 to localize to the body. p53 mutants L344A and L348A/L350P failed to localize to the body; thus oligomerization and nuclear export of p53 are important for its localization to the body. Although E1B body forms in the absence of p53, coexpression of p53 and E1B enhanced its formation. Thus p53 may stabilize E1B body. Two point mutants of Ad12 E1B, R225A and H245A, whose equivalent residues of Ad5 E1B are required for binding to p53, exhibited different properties: the former affected neither formation of the body nor the localization of p53 to it. In fact, R225A formed more elongated body. In contrast, E1B H245A failed to form the cytoplasmnic body. Cellular proteins are suggested to assist the localization of p53 to the E1B body, but absence of *MDM2* or *MDM4* neither affected the formation nor the sequestration of p53 in the body, although MDM2 binds to the same p53 peptide. Attachment of p53 aa 11-27 to p73 that does not interact with E1B resulted in sequestration of p73 in the body and this did not affect the transcriptional potential of p73. Interestingly, although such sequestration strongly stabilized p73, it markedly reduced p73-induced apoptosis. Surprisingly, our data revealed that Ad12 E1B inhibited wild-type p73-mediated apoptosis in the absence of physical interaction, probably through inhibiting an event downstream of p73-mediated apoptotic mechanisms. Our data thus implicate a role for p73 in the induction of apoptosis that is independent of transactivation.

1398

### Repression of Human Telomerase Reverse Transcriptase using Artificial Zinc Finger Transcription Factors

J. Choi, J. Sohn, J. Yoon, H. Lee, B. Yeh, H. Kim; Department of Biochemistry and Institute of Basic Medical Sciences, Yonsei University Wonju College of Medicine, Wonju, Republic of Korea

Telomerase activation is a key step in the development of human cancers. Expression of the catalytic subunit, human telomerase reverse transcriptase (hTERT), represents the limiting factor for telomerase activity, and that the regulation of hTERT should mainly occur at the transcriptional level. In this study, we used artificial zinc finger protein (ZFP) transcription factors to repress expression of hTERT in human cancer cell lines. We created potent transcriptional repressors by fusing a designed ZFP targeted to the hTERT core promoter region. KRAB repressor domain fused artificial ZFPs that bind a 12-bp recognition sequence within the promoter of the endogenous hTERT gene gives a >10-fold reduction in luciferase reporter assay. We also observed that transfection of ZFP in telomerase-positive cells promotes the reduction of hTERT mRNA level and decreases telomerase activity. In conclusion, the repression of telomerase expression by artificial ZFPs targeting the promoter region of the hTERT presents a promising strategy for inhibiting the growth of human cancer cells.

1399

### Role of Actin Bundling Protein Fascin in Cancer Metastasis

D. Vignjevic,[1] K. P. Janssen,[2] G. Bousquet,[1] S. Robine,[1] D. Louvard[1]; [1]Institute Curie, Paris, France, [2]Klinische Forschergruppe, Technische Universitaet, Muenchen, Germany

Cancer cells become metastatic by acquiring a motile and invasive phenotype. This step requires remodelling of the actin cytoskeleton and the expression of exploratory, sensory organelles known as filopodia. Previously, it has been tested whether efficient bundling of actin filaments within filopodia, is critical for filopodia formation. Fascin was the only actin bundling protein found to be localized along the whole length of all filopodia. Targeted depletion of fascin by siRNA lead to the substantially reduced number of filopodia. Because of their structure, filopodia can easily infiltrate between cells and by identifying appropriate targets for adhesion, generate traction forces to move the cell body. Fascin may promote metastasis, by participating in the formation of filopodia. Using qRT-PCR and immunohistochemistry, we found that fascin expression was significantly increased in human and mouse intestinal tumors compared with normal epithelium, in tumor stage depended manner. Expression was unchanged in benign lesions and early tumor stages, but significantly upregulated in invasive and metastatic lesions Next, we tested weather fascin can promote invasion and migration of human adenocarcinoma cells HT29: 1) in vitro, in trans-filter assays and 2) in vivo, by tail vein injection of cells into SCID mice. Fascin expressing cells had higher migrational potential in vitro; and in vivo led to more frequent distant metastasis in the lungs. Moreover, mice injected with fascin positive cells developed severe paralysis. Paralysis was caused by development of tumors along the spine, which invaded a back muscle. Those tumors were positive for villin (enterocytes marker) and fascin, which confirmed that they derived from the injected cells. We propose that up-regulation of fascin, by promoting the formation of filopodia, could be a significant component in the acquisition of invasive phenotype in carcinomas.

1400

### Role of Rho-like Proteins of the RhoBTB Family in Oncogenesis

J. Berthold,[1] S. Ramos,[1] M. Kopp,[1] P. Aspenstrom,[2] Y. Xiong,[3] P. Zigrino,[4] C. Mauch,[4] F. Rivero[1]; [1]Center for Biochemistry I, University of Cologne, Cologne, Germany, [2]Ludwig Institute for Cancer Research, Biomedical Center, Uppsala, Sweden, [3]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, [4]Department of Dermatology, University of Cologne, Cologne, Germany

Rho proteins have been implicated mainly in the regulation of the cytoskeleton, but also in vesicle trafficking, phagocytose and transcriptional activation. They also participate in tumorigenesis, where some of them are overfunctional. A subfamily of Rho GTPases, RhoBTB proteins, is represented by three isoforms in vertebrates. They consist of a GTPase domain, a proline-rich region and two BTB domains. BTB domains are involved in the formation of cullin 3-dependent ubiquitin ligase complexes. RhoBTB2/DBC2 has been proposed as a candidate for a tumor suppressor gene involved in breast cancer. We investigate the role of RhoBTB proteins in oncogenesis and the signal transduction pathways in which these proteins are involved. Using two-hybrid and co-immunoprecipitation studies we found that RhoBTB2 and 3 interact specifically with cullin 3 through both BTB domains. We also show that RhoBTB2 and RhoBTB3 homo- and heterodimerize. The ring box protein Roc1 enhances the interaction between RhoBTB and Cullin 3. RhoBTB3 might also be degraded in the proteasome: cells transfected with RhoBTB3 and treated

with a proteasomal inhibitor showed a clear accumulation of the protein. Furthermore, we found that RhoBTB3 interacts with ubiquitin conjugating enzyme. To address the role of RhoBTB in tumorigenesis we analyzed the expression profile of RhoBTB genes using a cancer profiling array. RhoBTB expression was decreased in all kidney samples and 80% of breast samples versus their matched normal tissue. For 50 cases of breast and 20 of kidney significantly decreased expression of RhoBTB1 and RhoBTB3 versus the matched normal tissue was observed. The expression change of RhoBTB3 and Cullin 3 correlates. We envision a model in which RhoBTB proteins target specific substrates for degradation by formation of cullin complexes and suppression of RhoBTBs results in accumulation of the target followed by cell proliferation.

1401

**Intracellular Trafficking, Cell Surface Protein Interactions and Regulated Cleavage of Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF) in Polarized Epithelial Cells**

A. H. Braun,[1,2] J. L. Franklin,[1] R. J. Coffey[1]; [1]Gastrointestinal Cancer and Epithelial Cell Biology, Vanderbilt University, Nashville, TN, [2]Internal Medicine (Cancer Research), West German Cancer Center, University of Duisburg-Essen, Germany

The EGF receptor (EGFR) is an important regulator of multiple cellular processes from embryonic development to epithelial tissue homeostasis and carcinogenesis. EGFR ligands are synthesized as transmembrane precursors that are targeted to specific cell surface compartments, from which mature soluble ligands are released through proteolytic cleavage of their ectodomains. The regulation of ligand trafficking, processing and ectodomain shedding is critical for their biological function. We have investigated the targeted delivery, cell surface protein interactions and regulated cleavage of HB-EGF in polarized MDCK II cells. HB-EGF is detected predominantly at the basolateral membrane (immunochemistry), but is mainly released from the apical surface in a metalloprotease-dependent manner (data generated with a newly-developed HB-EGF ELISA). Metabolic-labeling and pulse-chase analyses, as well as studies with non-cleavable mutant HB-EGF, confirm these observations (confocal immunofluorescence and selective cell-surface biotinylation). Seven-membrane-spanning receptor (LPA, bombesin) or PMA stimulation may induce recruitment of metalloproteases and/or their activators into HB-EGF complexes, resulting in regulated release of HB-EGF. Data on proteins interacting with full-length or non-cleavable HB-EGF as well as HB-EGF's cytoplasmic tail will be presented (mass spectrometry). Amongst metalloproteases implicated in HB-EGF cleavage, we have identified a role for ADAM-10/kuzbanian, ADAM-17/TACE and MMP-7/matrilysin by pharmacological (Way-022, Ex-01) or siRNA blockade and exogenous expression (ADAM-10, ADAM-17, MMP-7) in a battery of human breast and colorectal cancer cell lines. The expression of HB-EGF in normal and neoplastic epithelial tissue as detected with antibodies recognizing N- or C-terminal domains will be shown and correlated to the expression of ADAM-10 and -17. Differential expression and processing of HB-EGF and metalloproteases implicated in its cleavage in normal versus malignant tissue indicate that HB-EGF-related events are promising targets for cancer therapy.

1402

**Heterologous Expression and Characterisation of the *Agaricus bisporus* Lectins Which Inhibit *in vitro* Proliferation of Human Colon Cancer Cells**

J. Henderson,[1] B. Herman,[2] D. Eastwood,[2] D. West,[1] S. Sreenivasaprasad,[2] K. Burton[2]; [1]School of Science and the Environment, Coventry University, Coventry, United Kingdom, [2]Warwick HRI, Warwick University, Coventry, United Kingdom

*Agaricus bisporus* is an economically important edible fungus. Despite its importance, the *A. bisporus* genome has remained underexploited. Previous studies have identified a lectin from *A. bisporus* which inhibits epithelial colon cancer cell growth. Colorectal cancer is one of the second most common cause of cancer-related deaths in he Western world with 28,000 cases per year in the United Kingdom. The *A. bisporus* lectin is part of a group of proteins that can bind to the Thomsen-Friedenreich antigen (T0antigen) which is over-expressed in malignant and premalignant colonic epithelium. This T-antigen consists of the disaccharide sialyl-2, 3-galactosyl-β1-3-N-acetyl-galactosamine-α (Galβ1-3GalNAc). Binding of the *A. bisporus* lectin causes dose-dependent inhibition of the proliferation of these cells and is a reversible, non-cytotoxic effect. Recently, it has been shown that the lecin blocks nuclear localisation signal dependent nuclear protein import. Up to five isoforms the lectin have been detected in crude mushroom extract. It is yet unknown if these isoforms are formed by post-translational modification of a single lectin or whether there are more than one lectin gene involved or, it is a combination of both possibilities. In orer to test the functionality of the different lectin isoforms and to unveil the mechanism of action of the anti-proliferative effect it is essential to have access to high quantities of the purified single lectin(s). The work presented deals with the cloning and characterisation of lectin genes in *A. bisporus* and their heterologous expression.

1403

**The Antitumoral and Biochemical Effects of Gossypol on Human Cervix Cancer Cell Line ME-180**

E. Simsek, E. Arslan Aydemir, K. Fiskin; Biology, Akdeniz University, Antalya, Turkey

Gossypol, a yellow polyphenolic compound extracted from cotton plants, has been found to be a contraseptive, an antimicrobial, anti-viral, anti-parasitic and anticancerogenic agent. Recently, gossypol has been shown to inhibit the growth of several tumour cell lines and has been suggested to be a potential anticancer drug. The antitumour activity of gossypol has been reported in many previous studies. The aim of our study was to investigate whether gossypol induced cell death by DNA fragmentation on ME-180 cervix cancer cells; and whether it was a potent inhibitor of some antioxidant enzymes; like catalase, glutathione reductase and glutathione-S-transferase. Firstly, we investigated the effects of gossypol on growing tumour cells. The cells were incubated with four different doses (5,10,15 and 20µM ) for 24, 48 and 72 hours. After gossypol treatment, the cytotoxic effects were measured with MTT tests. Using DNA agarose gel electrophoresis, cellular internucleosomal DNA fragmentation of the cells treated with gossypol and untreated were examined. Consequently, gossypol caused different fragmentation on tumour cells due to apoptosis. After gossypol administration, different levels of activities were found in the enzymes of the control and treated cells. Gossypol was found to be a potent inhibitor of catalase, glutathione reductase and glutathione-S-transferase. Gossypol could realise this activity accompanied with dose and time dependent manner. Here, it is shown that treatment of ME-180 cells with gossypol not only induces apoptosis , but also a potent inhibitor of this three enzymes. The research results have been appraised by statistical analysis, compared with other research results.

1404

**Nuclear Interactions of Topoisomerase II and Phospholipid Scramblase 1**

J. P. Wyles, Z. Wu, S. E. L. Mirski, S. P. C. Cole; Cancer Biology and Genetics, Queen's University, Kingston, ON, Canada

DNA Topoisomerase (topo) II is a nuclear enzyme essential for cell division and is a well established target for several clinically effective chemotherapeutic agents. All vertebrates have two isoforms of topo II (α and β), and although they are highly homologous and both catalyze the same DNA cleavage, strand passage and DNA reunion reactions, they are differentially regulated and have distinct cellular functions and drug interactions. Most of the sequence variations between topo IIα and β are found in their COOH-terminal domains (CTDs), suggesting that these regions may be responsible, at least in part, for their functional differences. To better understand the basis for the different properties of topo IIα and β, yeast two-hybrid analysis was used to identify protein partners of their CTDs. One of the proteins identified by this approach was phospholipid scramblase 1 (PLSCR1). The interactions of PLSCR1 and topo II were further confirmed by (i) GST pull-down assays with GST-topo IIα and β CTD fusion proteins and detergent-solubilized endogenous PLSCR1; and (ii) co-immunoprecipitations of endogenous PLSCR1 and endogenous full-length topo IIα and β in HeLa cell nuclear extracts. Subsequent GST pulldown assays defined a conserved region of topo IIα (residues 1431-1441, GAKKRAAPKGT) as mediating binding to PLSCR1. PLSCR1 is typically regarded as a plasma membrane protein that plays an important role in apoptosis. However, a fraction of PLSCR1 is located in the nucleus and has been implicated in cellular differentiation and proliferation, as have both isoforms of topo II. Our studies reveal for the first time a potential role for PLSCR1 in topo II-mediated cell differentiation and proliferation pathways. Supported by the Canadian Cancer Society through the National Cancer Institute of Canada. JPW is the recipient of a Terry Fox NCIC Postdoctoral Fellowship.

1405

**Alteration of Calcium Signaling of Primary Cultured Keratinocytes in SERCA2$^{+/-}$ Mice**

J. Hong,[1,2] D. Shin,[1] H. Byun[1]; [1]Dept of Oralbiology, Yonsei University, Seoul, Republic of Korea, [2]Brain Korea 21 project, Seoul, Republic of Korea

The calcium homeostasis is connected to various cell functions that are involved in intracellular signal transduction. The sarco/endoplasmic reticulum $Ca^{2+}$-ATPases (SERCAs), plasma membrane $Ca^{2+}$-ATPases (PMCAs), and inositol trisphosphate receptors ($IP_3Rs$) are controlling $Ca^{2+}$ gradients in cellular membrane. This mechanism of SERCAs, PMCAs, and $IP_3Rs$ is essential for $Ca^{2+}$-mediated signaling and other biological processes. Moreover, free calcium ion has been served as a modulator of keratinocyte for proliferation and differentiation in mammalian skin. We found that SERCA2 heterozygous mutant mice (SERCA2$^{+/-}$) showed a very high rate of squamous cell carcinoma. It occurred to mice which are from 53 to 81 weeks. However, it is unknown that precise cause-effect relationship between $Ca^{2+}$ signaling and squamous cell carcinoma. In this study, we investigated $Ca^{2+}$ signaling in primary cultured murine epidermal keratinocyte from SERCA2$^{+/-}$ mice. Although there was no difference of the $[Ca^{2+}]_i$ entry in the wild type and the SERCA2$^{+/-}$ mice, SERCA2$^{+/-}$ mice appeared low initial $[Ca^{2+}]_i$ increasing in response to uridine 5'-trisphosphate, a potent $Ca^{2+}$-mobilizing agent, and sustained $[Ca^{2+}]_i$. In addition, SERCA2$^{+/-}$ mice seemed to more increased $[Ca^{2+}]_i$ than wild type mice, after depletion of intracellular $Ca^{2+}$ stores with cyclopiazonic acid (CPA), a specific inhibitor of SERCA pump. Western blot analysis revealed lower expressions of SERCA2, PMCA, and $IP_3R3$ in SERCA2$^{+/-}$ mice compared to wild-type mice respectively, but $IP_3R$ activity of SERCA2$^{+/-}$ mice is similar to wild type mice. The results indicated that $Ca^{2+}$ signaling and expression levels of $Ca^{2+}$-related proteins in primary cultured keratinocytes from SERCA2$^{+/-}$ mice were altered, but the precise mechanism of squamous cell carcinoma by decreased expression of SERCA2 will be further studied. This study was supported by a grant of the Ministry of Health & Welfare, Republic of Korea (A050002).

1406

**The Antitumoral and Biochemical Effects of Gossypol on Human Lung Cancer Cell Line H1299**

E. Aslan Aydemir, K. Fiskin, E. Simsek; Biology, Akdeniz University, Antalya, Turkey

Gossypol, a polyphenolic compound extracted from cotton plants, has been found to be a contraseptive, an antimicrobial and anticancerogenic agent. Recently, gossypol has been shown to inhibit the growth of several tumour cell lines and has been suggested to be a potential anticancer drug. The antitumour activity of gossypol has been reported in many previous studies. The aim of our study was to investigate whether gossypol induced cell death by DNA fragmentation on H1299 lung cancer cells and whether it was a potent inhibitor of some antioxidant enzymes; like catalase, glutathione reductase and glutathione-S-transferase. Firstly, we investigated the effects of gossypol on growing tumour cells. The cells were incubated with four different doses (5,10,15 and 20μM ) for 24, 48 and 72 hours. After gossypol treatment, the cytotoxic effects were measured with MTT tests. Using DNA agarose gel electrophoresis, cellular internucleosomal DNA fragmentation of the cells treated with gossypol and untreated were examined. Consequently, gossypol caused different fragmentation on tumour cells due to apoptosis. After gossypol administration, different levels of activities were found in the enzymes of the control and treated cells. Gossypol was found to be a potent inhibitor of catalase, glutathione reductase and glutathione-S-transferase. Gossypol could realise this activity accompanied with dose and time dependent manner. The research results have been appraised by statistical analysis, compared with other research results.

1407

**Consequences of Modulating *GLI-1* Expression in a Basal Carcinoma Cell Line**

P. Romagnoli,[1] R. Sestini,[2] P. Di Gennaro,[1] S. Bacci,[1] L. Domenici,[1] P. Pinzani,[2] P. Carli,[3] M. Genuardi[2]; [1]Department of Anatomy, Histology and Cell Biology, University of Florence, Firenze, Italy, [2]Department of Clinical Physiopathology, University of Florence, Firenze, Italy, [3]Department of Dermatological Sciences, University of Florence, Firenze, Italy

*GLI-1* is an oncogene amplified in human gliomas and several other tumours, which encodes a transcription factor mediating the transduction of Sonic Hedgehog signal. The expression of *GLI-1* is typical of basal cell carcinoma and is supposed to be responsible for increased proliferation rate, expression of the antiapoptotic protein BCL-2 and reduced adhesion to the basal lamina because of inhibited expression of laminin. These events may be relevant to tumour pathogenesis. Tacrolimus (FK506) is an immunomodulatory molecule with extensive homology with rapamycin; the latter can antagonize the cellular transformation caused by *GLI-1* overexpression, independent of immunomodulation. We tested whether tacrolimus was active on the expression of *GLI-1* and if so which were the consequences on downstream genes in the basal carcinoma cell line TE 354.T. *GLI-1* mRNA expression was estimated by real-time PCR with Taqman probes; BCL-2 and laminin were estimated by

immunohistochemistry and BCL-2 also by Western blot; basal lamina material was evaluated by electron microscopy; the proliferation index was estimated by bromodeoxyuridine incorporation and apoptosis was estimated by TUNEL assay. The cell line under study expresses *GLI-1* mRNA and expresses BCL-2 and laminin proteins with variable intensity among cells. Tacrolimus caused partial inhibition of *GLI-1* transcription, accompanied by reduced expression of BCL-2, increased expression of laminin, and increased secretion of basal lamina material. Tacrolimus did not influence the cell growth rate, proliferation index, nor apoptosis. The results support the hypothesis of a role of *GLI-1* in the regulation of the expression of BCL-2 and in the secretion of basal lamina proteins by basal carcinoma cells and lead to propose tacrolimus as an efficient tool to study the effects of inhibiting *GLI-1* expression in cells spontaneously expressing this transcription factor.

1408

**Induction of Synthesis of Metallothionein by Platinum Based Drugs**

R. Prusa,[1] J. Petrlova,[2] O. Blastik,[2] V. Adam,[2] J. Kukacka,[1] R. Kizek[2]; [1]Clinical Biochemistry and Pathoiochemistry, Charles University, 2nd Medical Faculty, Prague 5, Czech Republic, [2]Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Brno, Czech Republic

Neoplastic diseases represent one of the most prevalent human disorders. Many of those are generally treated by platinum-complexes. Effectiveness of such treatment depends on the therapeutic concentration of the drug commonly influenced by binding to proteins (e.g. metallothionein). The metallothionein (MT) family is class of low molecular, intracellular and cysteine-rich proteins presenting high affinity for metal ions. Metallothionein is a well-known heavy metal binding protein maintaining metal ion homeostasis. In human organisms MTs are usually abundant at low concentrations levels. Their marked expression is caused by many exogenous and endogenous factors such as UV radiation, heavy metals, xenobiotic species, reactive oxygen species etc. The aim of this work was to study the interaction of MT with Pt compounds. The proper interaction was observed *in vitro* by means of electrochemical techniques using the oxidation-reduction and/or catalytic signals (Brdicka reaction, and peak H). Chronopotentiometric stripping analysis was the most sensitive and, therefore, the most suitable technique for studying of interaction between platinum and MT. The interaction was observed by means of changes in electrochemical signal (peak H) of MT on the surface of hanging mercury drop electrode. Finally, the suggested technique was used for analysis of blood serum of the patients treated with standard platinum compounds. The MT amount increased in this patients´ serum more than 10 times in comparison with non-treated patients. The results thus show that the MT amount has a significant negative influence on the therapy of neoplastic diseases. **Acknowledgements.** *This work was supported by grants: GACR 525/04/P132 and RASO 2005.* **References** [1] V. Adam, et. al. 99 (2005) 353. [2] R. Prusa, et. al. Clin. Chem. 51 (2005) A56. [3] R. Prusa, et. al. Clin. Chem. 50 (2004) A28. [4] R. Kizek, et. al. Anal. Chem. 73 (2001) 4801.

1409

**Ligand Activation of FGFR-1 Isoforms Induces Differential Signaling Responses**

P. Zhang,[1] J. A. Thompson[2]; [1]Dept of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL, [2]Dept of Surgery, Dept of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL

Overexpression of FGF ligands and the alternative spliced FGFR-1β isoform have been found in malignant pancreatic adenocarcinoma, the fourth leading cause of cancer death in US. In normal adult tissue FGFR-1 is typically expressed as the α isoform. To examine the biological significance of this feature, FGF receptor-negative endothelial cells (RVEC) were stably transfected with expression vectors encoding either FGFR-1α or FGFR-1β human cDNA. In the presence of FGF-1 treatment, these two isoform transfectants exhibited differential phenotype, migratory behavior, and responses to oxidant stress, ionizing radiation and chemotherapy. These observations have prompted us to examine the intracellular substrates activated by the two receptor isoforms. Immediate early, transient activation of c-YES and FAK were limited to FGFR-1β signaling and correlated with altered phenotype, increased migration, and resistance to cytotoxic treatments. Activation of FRS2, SHC and N-Ras were identified in both isoforms, resulting in sustained activation of ERK1/2 MAPK and mitogenic behavior. Immediate early, transient activation of the stress-associated MAPKs, p38 and JNK, was restricted to FGFR-1α signaling in an extracellular calcium- and PKC-dependent manner. Ligand-activated FGFR-1α transfectants demonstrated increased sensitivity to cytotoxic treatments. Taken together, these differential signaling pathways may provide mechanistic insight into the onset, progression, and treatment of tumorigenesis.

1410

**Study of Binding of Platinum Cytostatic Drug to DNA Structure: Therapeutic Concentration of Platinum Cytostatic**

R. Prusa,[1] V. Adam,[2] J. Petrlova,[3] J. Zehnalek,[3] R. Kizek[3]; [1]Clinical Biochemistry and Pathoiochemistry, Charles University, 2nd Medical Faculty, Prague 5, Czech Republic, [2]Anylytical Chemistry, Masaryk University, Faculty of Science, Brno, Czech Republic, [3]Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Brno, Czech Republic

Although cisplatin, cis-diamminodichloroplatinum(II), has been successfully used in the chemotherapy of cancer for more than 25 years, its biochemical mechanism of action is still unclear. The aim of this paper was to study of binding of platinum cytostatic to DNA structure using of platinum biosensor. Moreover, we applied the biosensor to analyse platinum in human blood serum - therapeutic concentration assay. Metallothionein modified hanging mercury drop electrode (HMDE) surface was used as a platinum biosensor. The platinum and Pt-DNA adduct were detected by adsorptive transfer stripping (AdTS) differential pulse voltammetry (DPV). It is necessary for the study of the anti-cancer drugs effectiveness to detect their therapeutic level. That is why we applied the MT modified HMDE for determination of *cis*-platin in the human blood serum. The detection limit (3 S/N) of cisplatin was about 10 pmole in 5 µl. In addition therapeutic effect of cisplatin relates to conformational changes in the DNA structure. The interaction between Pt(II) and DNA probably proceeds via guanine (G) molecules. Here, we used the biosensor to study of amounts of Pt bound to DNA. Detection limit of MT biosensor calculated for Pt-DNA adduct was hundreds of nanograms per millilitre. The suggested approach shows the possible way for simple, sensitive and rapid detection of cisplatin and Pt-DNA in the human body fluids at the picomole level. The biosensor could be applied to study of binding of platinum to DNA structure. **Acknowledgements.** *This work was supported by grants: GA CR No. 525/04/P132 and RASO 2005.* **References** [1] R. Kizek et. al.: Anal. Chem. 73 (2001) 4801. [2] R. Prusa et. al.: Clin. Chem. 50 (2004) A28. [3] E. Palecek et. al.: Anal. Chem. 76 (2004) 5930.

## Metabolic Diseases (1418-1442)

1411

### DHEA-induced Antiproliferative Effect in MCF-7 Cells is Androgen- and Estrogen- Receptor Independent

R. López Marure, V. Gayosso Tellez, L. F. Montaño Estrada; Fisiología, Instituto Nacional de Cardiología, México D. F., Mexico

Dehydroepiandrosterone (DHEA), an adrenal hormone derived from cholesterol, can be metabolized to estrogens (estradiol) and androgens (testosterone). In this work, we evaluated if the antiproliferative effect induced by DHEA in MCF-7 cells is direct, or indirect through its conversion to estradiol or testosterona. Cell proliferation was evaluated by violet crystal staining and the androgen and estrogen receptors were blocked by ICI 182,780 and flutamide inhibitors. DHEA had an antiproliferative effect at supraphysiological concentrations, however when it was used at physiological concentrations, it increased the proliferation of MCF-7 cells. Estradiol induced an increase on the cell proliferation, at physiological concentrations, whereas testosterone had a weak inhibitory effect at 100 μM. DHEA-S (its inactive sulfate ester) had no effect upon the cell cycle. DHEA-induced antiproliferative and proliferative effect was not blocked by inhibitors of androgen or estrogen receptors, thus indicating that its effect is secondary to a direct interaction with a "putative" receptor rather than conversion into steroid hormones. These results suggest that DHEA could be used at supraphysiological concentrations in the treatment of breast cancer.

1412

### Antisense Experiments Demonstrate Cancer-Specific Encoding of Functional tNOX Through an Exon 4 Minus Splice Variant

X. Tang,[1] D. Morre,[2] D. M. Morre[1]; [1]Foods and Nutrition, Purdue University, West Lafayette, IN, [2]Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN

Real-time quantitative PCR and in vitro cytotoxicity assays were combined to demonstrate encoding of functional tNOX, a cancer specific and growth-related cell surface protein, via an Exon 4 minus splice variant. Total RNA from HeLa cells treated with antisense oligonucleotides for 6 h was isolated followed by measurement of specific tNOX mRNA using real-time quantitative RT-PCR. Cells were transfected with oligonucleotides using Lipofectamine 2000 transfection reagent. tNOX mRNA levels of each sample were calculated relative to control cells transfected with Lipofectamine and determined by the cycle threshold method normalized for GAPDH mRNA. Antisense to tNOX Exon 4 mRNA blocked the generation of full-length tNOX mRNA but not Exon 4 minus mRNA. An oligonucleotide with the scrambled sequence was without effect. Antisense to Exon 5 mRNA did inhibit the production of both Exon 4 minus mRNA and full length tNOX mRNA. Again, scrambled antisense to Exon 5 mRNA was without effect. Antisense to Exon 5 mRNA decreased the amount of tNOX protein on the surface of cancer cells. As a control, antisense mediated down-regulation of tNOX Exon 5 minus mRNA also was demonstrated as detected using Exon 4/Exon 6 primers. Exon 5 antisense blocked the cell surface expression of tNOX whereas Exon 4 antisense was without effect. Additionally, HeLa cells transfected with Exon 5 antisense were no longer inhibited by the green tea catechin tNOX inhibitor (-)-epigallocatechin-3-gallate. A relationship of tNOX to unregulated growth of cancer cells was provided by data where growth of HeLa cells was inhibited by transfection with the Exon 5 antisense oligonucleotides. Growth inhibition was followed by apoptosis in greater than 80% of the population of transfected cells.

1413

### Elucidation of Novel Mechanisms that Control Metastatic Cell Growth

G. S. Goldberg,[1] Z. Jia,[1] Y. Shen,[1] H. Ichikawa[2]; [1]Molecular Biology, UMDNJ, Stratford, NJ, [2]Cancer Transcriptome Project, National Cancer Center Research Institute, Tokyo, Japan

Anchorage independence and motility are hallmarks of metastatic tumor cell growth. Tumor cell growth and morphology can be normalized by contact with nontransformed cells. The Src tyrosine kinase phosphorylates specific sites on the focal adhesion adaptor protein Cas to promote nonanchored cell growth and migration. We studied the effects of Src and Cas on the expression of over 14,000 genes to identify molecular events that underlie these activities. Gene expression in tumor cells that were normalized by neighboring nontransformed cells was used as an additional filter to identify genes that control metastatic cell growth. This process enabled the identification of genes that appear to play roles in anchorage independent cell growth and migration. By independently investigating (1) anchorage independent cell growth, (2) migration, and (3) the effects of neighboring cells on tumor cells, we have developed an efficient method to identify novel mechanisms that control fundamental aspects of cell growth and metastasis.

1414

### Biological and Biochemical Characterization of a Novel Dual-Specificity Phosphatase, Neuroendocrine-Associated Phosphatase (NEP)

J. Wang,[1] C. Lin,[1] C. Yang,[1] T. Tan,[2] Y. Chen[1]; [1]Division of Molecular and Genomic Medicine, National Health Research Institutes, Miaoli County, Taiwan Republic of China, [2]Department of Immunology, Baylor College of Medicine, Houston, TX

A novel neuroendocrine-associated phosphatase (NEP) was characterized for its biochemical and biological properties. NEP showed a sequence characteristic of dual-specificity phosphatase (DSP) and was preferentially expressed in neuroendocrine cells/tissues as well as in skeletal muscle and heart. Expression of NEP was not significantly induced by oxidative stress or serum but was upregulated in PC12 cells during nerve growth factor (NGF)-induced differentiation. NEP was a cytosolic protein and *in vivo* did not have significant effects against activation of ERK, JNK, and p38-MAPK induced by various stimuli. Although NEP and mitogen-activated protein kinase (MAPK) phosphatase-1 (MPK-1) showed similar phosphatase ability toward *p*-nitro phenylphosphate (*p*NPP), NEP did not evidently dephosphorylate MAPKs as did MKP-1 *in vitro*. Our results indicated that, unlike other DSPs, down-regulation of MAPKs was not the major function of NEP. Furthermore, NEP induced by NGF during PC12 cell differentiation may be involved in NGF/TrkA signaling pathway.

1415

### The Role of GlUT1 in HTLV Infection

I. M. Lisinski, N. Takenouchi, F. Kazunori, K. Yao, F. W. Ruscetti, K. Jones, S. W. Cushman, S. Jacobsen; NIDDK, NIH, Bethesda, MD

GLUT1 was recently described as a receptor for the retrovirus HTLV. The entry of retroviruses into target cells involves the interaction of two envelope glycoproteins, a surface glycoprotein (SU) and a transmembrane glycoprotein (TM), with at least one specific host cell receptor. To

determine the importance of GLUT1 in virus infection, we first established a system that would allow us to over express GLUT1 in target cells, and monitor the relative amount of GLUT1 expressed on the cell surface. We transfected COS cells with GLUT1 and a mutant form of GLUT6 [GLUT6 (LL/AA)], both tagged with an hemagglutinin (HA) epitope to determine cell-surface expression by FACS. Quantitative Western blot analysis of bis-glucose photolabeled cells was also used to measure cell-surface expression. First we show that GLUT1 cell-surface expression is almost undetectable in untransfected, compared to GLUT1 over-expressing, COS cells. Cell-surface expression of mutant GLUT6 (LL/AA), but not wt GLUT6, is also similar to that of GLUT1 in transfected COS cells. Second, we demonstrate that over expression of GLUT1 does not increase HTLV SU binding suggesting that virion binding reflects interactions with cell-surface proteins other than GLUT1. Finally, to study the role of GLUT1 in HTLV-1 infection, we developed a co-culture assay, using live HTLV-1 producing cells, to monitor *de novo* infection of GLUT1 over expressing COS cells. In this case, the percentage of infected COS cells is drastically increased with HA-GLUT1-transfected cells (46.4%) compared to non-transfected and HA-GLUT6 (LL/AA)-transfected cells (2.0%). Thus, GLUT1 appears to have an important role in HTLV infection, but not binding; we propose that GLUT1 mediates vesicular uptake of bound virus through its subcellular trafficking properties.

1416

**Adenosine Receptor $A_3R$ Agonist Inhibits Cholangiocarcinoma Growth via Galpha$_i$-dependent Activation of $IP_3$ with Associated Downregulation of cAMP, Erk1/2 and Akt**

S. S. Glaser,[1] H. Francis,[1] S. DeMorrow,[1] J. Venter,[2] R. Reichenbach,[3] R. Summers,[2] B. Green,[2] A. Coots,[2] Y. Ueno,[4] G. Alpini[5]; [1]Research & Education, Department of Medicine, Texas A&M University HSC and Scott & White Hospital, Temple, TX, [2]Department of Medical Physiology, Texas A&M University HSC, Temple, TX, [3]Central Texas Veterans Health Care System, Temple, TX, [4]Division of Gastroenterology, Tohoku University School of Medicine, Sendai, Japan, [5]Department of Medical Physiology & Internal Medicine, Texas A&M University HSC, Scott & White Hospital and Central Texas Veterans HCS, Temple, TX

Cholangiocarcinoma is an aggressive biliary tumor, which retains many of the regulatory features controlling normal cholangiocyte proliferation. We have shown that activation of $IP_3/Ca^{2+}$/PKCα-dependent signaling inhibits cholangiocyte growth via downregulation of the cAMP/ERK1/2 pathway. We have previously demonstrated that the cholangiocarcinoma cell line, Mz-ChA-1, expresses all four adenosine receptor (AR) subtypes ($A_1R$, $A_{2A}R$, $A_{2B}R$ and $A_3R$) and that specific adenosine receptor agonists inhibit MzChA-1 growth to varying degrees. AR agonists have been reported to have dual signaling mechanisms via activation of $G\alpha_{i/o}$ resulting in decreased cAMP levels and increased $IP_3$ via stimulation of phospholipase C. The AIM of our study was to further evaluate the signaling mechanisms governing the inhibition of cholangiocarcinoma proliferation via stimulation of $A_3R$. Methods: Proliferation was evaluated by MTS assay. Mz-ChA-1 cells were stimulated with Cl-IB-MECA ($A_3R$ agonist, 1 nM) in the presence/absence of VUF5574 (a $A_3$ receptor antagonist, 1μM) and BAPTA/AM (intracellular $Ca^{2+}$ chelator, 5 μM) for 48 hours and proliferation was evaluated. Intracellular $IP_3$ and cAMP levels were determined by RIA following treatment of Mz-ChA-1 cells with Cl-IB-MECA in the presence/absence of pertussis toxin pretreatment. The effect of Cl-IB-MECA on ERK1/2 and AKT phosphorylation was determined by immunoblotting. Results: Cl-IB-MECA inhibited Mz-ChA-1 cell proliferation (30%), which was blocked by VUF5574 and BAPTA/AM. Cl-IB-MECA increased intracellular $IP_3$ levels, which was inhibited by pertussis toxin pretreatment. Cl-IB-MECA inhibition of cAMP levels was reversed by pertussis toxin. The phosphorylation of both ERK1/2 and AKT was significantly reduced following Cl-IB-MECA treatment. Conclusion: Simulation of $A_3R$ decreased proliferation of Mz-ChA-1 cells via a pertussis toxin sensitive $IP_3$-dependent mechanism with associated downregulation of the cAMP/ERK1/2 and AKT pathways. $A_3R$ agonists may represent a new therapeutic option to regulate cholangiocarcinoma growth.

1417

**Positive Regulation of p53 by Tumor Suppressor pVHL**

J. Roe, H. Youn; Seoul Nat'l Univ. College of Medicine, Seoul, Republic of Korea

The tumor suppressor p53 is activated in response to many types of cellular and environmental insults. The level of p53 is governed largely by its major negative regulator, Mdm2. Also, another tumor suppressor von-Hippel landau(VHL) has emerged as a key factor in cellular responses to oxygen availability. pVHL is required for the oxygen-dependent proteolysis of α subunit of hypoxia-inducible factor-1(HIF-1α). In many renal cell carcinoma patients, it is reported that VHL mutations are directly linked to carcinogenesis. Here we found that α domain of pVHL directly binds to p53 and this interaction prevents Elongin C recruitment to pVHL, so ubiqutin ligase activity of pVHL is disrupted. Also, this interaction is both HIF-1α and Mdm2 independent manner. In contrast to the known role for HIF-1α degradation, pVHL protects p53 from proteasomal degradation and enhances transcriptional activity. These phenomenon is supported by the fact that pVHL assists the recruitment of coactivators like p300 or pCAF. Interestingly, pVHL contributes to p53 dependent cell-cycle arrest at G1-phase and apoptotic cell death induced by DNA damaging agent. Both apoptosis and cell-cycle arrest caused by DNA damage are significantly attenuated in VHL null cell. These results suggest that pVHL participates in p53-medeatied signaling pathways and elucidates cancer formation in pVHL disrupted renal cells.

1418

**Identification of Novel Adipocyte-Secreted Factors Using Microarray Data of Various Metabolic Model Mice**

H. S. Jo, H. W. Jeong, J. B. Kim; Biological Science, Seoul National University, Seoul, Republic of Korea

Since the discovery of leptin in the mid 90's, the list of adipocyte-secreted factors has vastly expanded. An increasing body of evidence suggests that these factors, now referred to as adipocytokines, play critical roles in maintaining whole body energy homeostasis. Tissue specific knockout or overexpression of certain genes in adipocytes, such as IR, PPARγ, GLUT4, G6PD, and FOXC2, leads to aberrant changes in muscle and liver metabolism, affecting insulin sensitivity and susceptibility to the onset of diabetes. These effects are thought to be mediated, in part, by altered adipocytokine expression. To identify novel adipocytokines, we compared microarray data from various mouse models, including the ob/ob and db/db mice, and Rosiglitazone treated mice. We have discovered 11 candidate genes that have been previously cited and 1 gene encoding a hypothetical secreted protein. [This work was supported in part by grants from the National Research Laboratory Program of Korea Science and Engineering Foundation.]

1419

**Regulation of PPARg Protein Expression by Free Fatty Acids in 3T3-L1 Adipocytes**

A. Nguyen,[1] D. D. Sears,[1] H. Satoh,[2] J. M. Ofrecio,[1] J. M. Olefsky[1]; [1]Division of Endocrinology/Metabolism, University of California, San Diego, La Jolla, CA, [2]Fukushima Medical University, Fukushima-City, Japan

Regulation of PPARg Protein Expression by Free Fatty Acids in 3T3-L1 Adipocytes MT Audrey Nguyen[1], Dorothy D. Sears[1], Hiroaki Satoh[2], Jachelle M. Ofrecio[1], Jerrold M. Olefsky[1]. [1]Division of Endocrinology/Metabolism, University of California, San Diego, La Jolla, CA, USA, [2] Fukushima Medical University, Fukushima-City, Japan PPARg is a nuclear receptor whose expression is crucial for adipogenesis and metabolism, and a high-affinity target for the thiazolidinediones or TZDs, a class of powerful antidiabetic drugs. In humans and rodents, PPARg protein level and activity are critical for its beneficial effects as an insulin-sensitizing transcriptional regulator. In this study, we investigated the effect of free fatty acids (FFAs), agents known to decrease insulin sensitivity, on PPARg1 and PPARg2 protein expression in 3T3-L1 adipocytes. Adipocytes were treated with a mixture of saturated and unsaturated FFAs at various concentrations and for various amounts of time, and PPARg protein levels were examined by immunoblotting. FFA treatment led to a decrease in PPARg1 and PPARg2 protein levels in a time and dose-dependent manner. This was paralleled by a time and dose-dependent decrease in PPARg mRNA levels as determined by semi-quantitative RT-PCR. When cells were pretreated with the inhibitor of transcription actinomycin D, the FFA-induced decrease in PPARg mRNA was partially blocked, suggesting involvement of both mRNA stability and transcriptional regulation. When de novo protein synthesis was inhibited with cycloheximide, the FFA-induced decrease in PPARg protein was not prevented. Similarly, inhibition of protein degradation using lactacystin and MG-132 did not block the decrease in PPARg protein, indicating that proteasome-dependent proteolysis is unlikely to contribute to decreased PPARg protein levels. Together the data suggest that FFAs downregulate PPARg protein levels primarily through inhibition of PPARg gene transcription.

1420

**HIV-1 Protease Inhibitors Impair Insulin Signaling and Suppress Nitric Oxide (NO) Production in Vascular Endothelial Cells: Protective Effect of Thiazolidinediones (TZD)**

K. Liu, D. Mondal, M. Ali, K. C. Agrawal; Pharmacology, Tulane University Health Sciences Center, New Orleans, LA

Inclusion of HIV-1 protease inhibitors (PIs) in highly active antiretroviral therapy (HAART) has significantly decreased mortality in HIV-1 infected patients. However, PI therapy can manifest insulin resistance syndrome (IRS), a risk factor for diabetes and atherosclerosis. PI-mediated dysfunctions in vascular endothelial cells (ECs) are not well understood. We have shown that PIs increase oxidative stress in ECs which enhance endothelial-leukocyte adhesion. Since endothelial oxidative stress is regulated by insulin-induced NO production via NO synthase (eNOS), we hypothesized that PIs may suppress NO production by inhibiting eNOS and that insulin sensitization via TZDs may provide protection. Human aortic endothelial cells (HAECs) were exposed (72 hr treatment with fresh PIs added every 24 hr) to PIs [nelfinavir (1-5 μM), atazanavir (1-5 μM), or indinavir (3-15 μM)] and NO production was monitored (DAF-FMDA fluorescence assay). Exposure to PIs, especially nelfinavir, decreased both basal (10-15 %) and insulin-induced (15-40 %) NO production, and coexposure with TZDs [troglitazone or rosiglitazone (0.1-0.25 μM)] inhibited this suppression. Insulin binding to its receptor (IR) activates insulin receptor substrates (IRS-1 and -2), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB or AKT), which leads to increased eNOS expression and activation. We therefore monitored whether nelfinavir affects insulin signaling in HAECs. Immunoblotting of cell lysates from PI-exposed HAECs indicated that nelfinavir exposure decreased phosphorylation of IRβ and IRS-1, and downstream activation of AKT, PI3K (p85) and eNOS. Simultaneous addition of TZDs provided significant ($p<0.05$) protection. These data suggest that TZDs may provide therapeutic benefits towards suppressing the vascular dysfunctions observed in HIV-1 infected individuals receiving PI containing HAART regimen.

1421

**Intrinsic Sorting Signals Required to Target Newly-synthesized IRAP into the Insulin-responsive GLUT4 Storage Compartment**

R. T. Watson, J. Hou, J. E. Pessin; Pharmacological Sciences, SUNY-Stony Brook, Stony Brook, NY

The glucose transporter 4 (GLUT4) and the insulin-responsive aminopeptidase (IRAP) display indistinguishable membrane compartmentalization and insulin-stimulated translocation properties. IRAP is a type II integral membrane protein with a short cytoplasmic region of 109 amino acids that encodes all the information necessary for insulin-responsive compartmentalization. IRAP is therefore an excellent model protein for studying the sorting processes responsible for targeting cargo molecules into the insulin responsive GLUT4 storage compartment. Immediately following biosynthesis, the EGFP-IRAP/WT reporter construct trafficked to the Golgi complex and was efficiently sequestered in perinuclear membrane compartments. At 3-6 h following biosynthesis, EGFP-IRAP/WT did not translocate to the cell surface in response to insulin. However, beginning at 6-9 h following expression, EGFP-IRAP/WT acquired insulin responsive compartmentalization and translocated to the plasma membrane following acute insulin stimulation. In contrast, the EGFP-IRAP/AA(76,77) construct, wherein the dileucine residues at position 76,77 were mutated to alanine, rapidly defaulted to the cell surface following biosynthesis. This trafficking pattern was indistinguishable from a control syntaxin 3 construct that also rapidly defaulted to the cell surface. In addition, coexpression of the VHS-GAT dominant-interfering domains of GGA strongly inhibited the ability of EGFP-IRAP/WT to acquire insulin-responsive compartmentalization at all time points following biosynthesis. However, the VHS-GAT construct did not block the constitutive trafficking of syntaxin 3 or EGFP- IRAP/AA(76,77) to the cell surface. Moreover, the EGFP-IRAP/WT and EGFP- IRAP/AA(76,77) constructs occupied morphologically distinct tubulovesicular carrier compartments in the perinuclear Golgi region. Taken together, the above data indicate that LL76,77 functions at the level of the Golgi complex to target newly-synthesized IRAP protein into the insulin-responsive GLUT4 storage compartment.

1422

**BLX-1028, a Novel Antiadipogenic Compound, Lowers Body Weight Gain in *ob/ob* and Diet Induced Obese Mouse Model**

S. Roy, H. Moon, M. Anamula, A. Sen, A. Nag, P. Neogi, B. Nag, D. Dey; Bexel Pharmaceuticals, Union City, CA

BLX-1028 is a water soluble, orally active, modified amino acid derivative with no structural similarity to any existing antiobesity compounds. It inhibits both PPAR(- and "Dexamethasone-Insulin-IBMX"- mediated adipocyte differentiation in 3T3-L1 fibroblasts. BLX-1028 dose dependently inhibits iNOS expression in both TNFα-induced adipocytes and LPS-induced macrophages. It has been shown that iNOS expression is upregulated in muscle and adipose tissues of both dietary and genetic obesity animal models and targeted disruption of iNOS protects against obesity-linked

insulin resistance in muscle. Since our compound shows a unique antiadipogenic and anti-inflammatory properties, following studies were undertaken to determine the body weight lowering effect of BLX-1028 in different mouse models. Obesity was induced in C57BL/6J mice by exposure to a high fat (60% Kcal fat) diet with free access to water. After 14 days of high fat diet feeding, animals were treated orally, once daily (1 hour before dark cycle) for 30 days with BLX-1028 (5mg/Kg body weight). Compared with placebo treatment, BLX-1028 lowered the body weight gain by 14% without significant changes in food and water intake. Similarly in *ob/ob* (C57BL/6J-Lep$^{ob}$) mice, 10% reduction in body weight was observed in BLX-1028 treated animals in comparison to vehicle treated groups. Oral Glucose Tolerance test performed at day 0 and 30 showed an improvement in glucose tolerance in these mice. Further mechanism of action of BLX-1028 is currently being investigated. These combined results demonstrate the potential of BLX-1028 as a novel compound for the treatment of obesity and related disorders.

1423

**Molecular Mechanisms Underlying Pancreatic β-cell Loss in a Murine Model of Wolfram Syndrome**

H. Ishihara, T. Yamada, D. Takei, Y. Oka; Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Sendai, Japan

Wolfram syndrome, a rare autosomal recessive disorder characterized by juvenile onset diabetes mellitus, optic atrophy, diabetes insipidus and sensorineural deafness, is caused by mutations in the *WFS1* gene, which encodes an ER resident membrane protein. We have recently established mutant mice with disrupted *wfs1* gene and found that mutant mice exhibited impaired glucose homeostasis due to progressive β-cell loss and impaired stimulus-secretion coupling in insulin secretion. To further investigate mechanisms of β-cell dysfunction, we have conducted studies of cellular physiology and gene expression, especially focusing on ER functions and ER-stress responses, in *wfs1*-deficient cells. ER calcium concentrations, measured using ER-targeted aequorin, were lower in the *wfs1*-deficient cells, which seemed to be attributable to reduced capacitative calcium entry. We also found that increased PERK phosphorylation and XBP-1 expression, indicating that the ER stress responses were enhanced in *wfs1*-deficient islets. The enhanced ER stress responses were accompanied by increased expression of proapoptotic protein CHOP and by an increase in the cleaved form of caspase-3, indicating that ER stress-mediated apoptotic process is initiated in *wfs1*-deficient β-cells. In addition, we found that BrdU incorporation was reduced in the mutant islets. Interestingly, expressions of cell cycle regulators and of the translational control machinery were altered. These data suggest that WFS1-deficiency causes impaired ER calcium homeostasis in β-cells, leading to chronic activation of the ER-stress response, which then eventually results in increased apoptosis and impaired proliferation. Our data thus establish that, as is the case in Wolcott-Rallison syndrome with PERK mutations, ER stress is the pathological basis of Wolfram syndrome.

1424

**Organelle Dynamics during Chronological Aging of Calorie-Restricted Yeast Cells**

A. Goldberg,[1] C. Gregg,[1] T. Guo,[1] T. Boukh-Viner,[1] S. Bourque,[1] P. Kyryakov,[1] M. Noei,[1] R. Noei,[1] A. Brar,[1] R. A. Rachubinski,[2] Y. Gélinas,[3] A. M. English,[3] O. I. Petriv,[1] V. I. Titorenko[1]; [1]Biology Department, Concordia University, Montreal, PQ, Canada, [2]Department of Cell Biology, University of Alberta, Edmonton, AB, Canada, [3]Department of Chemistry and Biochemistry, Concordia University, Montreal, PQ, Canada

Calorie restriction (CR), a dietary regimen low in calories without undernutrition, significantly extends life span and delays most diseases of aging including cancer, atherosclerosis, type II diabetes and neurodegeneration. Even though benefits of CR have been known for many years, the molecular and cellular mechanisms of its action remains unclear. Growing evidence supports the view that the fundamental mechanism of aging is conserved from yeast to humans. We use the yeast *Saccharomyces cerevisiae* as a model system with which to study the molecular and cellular mechanisms of aging. Our research is aimed at understanding how defects in the biogenesis and function of the peroxisome, an organelle known for its role in lipid metabolism, affect life span of yeast. We identified a distinct set of peroxisome-associated proteins that function as global regulators of longevity and stress response in yeast cells placed on the CR diet. Using functional proteomics, we defined a network of protein components whose synthesis and degradation are regulated by these peroxisome-associated proteins. Analysis of mutants that lack individual components of this network demonstrated that many of these components are key determinants of life span and stress resistance of yeast. Using lipidomics, we analyzed the chronological changes in steady-state levels of various lipid species in cells and subcellular organelles of numerous short-living mutants. Our data suggest a model for the hierarchy of events that occur during the remodeling of metabolic pathways and inter-organellar communications in yeast cells placed on the CR diet.

1425

**Generation and Verification of Functional Insulin-producing Cells Derived from Embryonic Progenitor Cells**

J. Zhang,[1] R. Luo,[1] O. L. Kon,[2] F. Xie,[1] S. K. Lim,[3] G. LI[1]; [1]National University Medical Institutes, National University of Singapore, Singapore, Singapore, [2]Division of Medical Sciences, National Cancer Centre, Singapore, Singapore, [3]Stem Cell Biology, Genomic Institute of Singapore, Singapore, Singapore

Transplantation of human pancreatic islets is an efficient therapy for treatment of type 1 diabetes. However, only limited number of patients could benefit from this promising remedy because of the shortage of cadaver pancreas. Therefore, generation of propagational, functional insulin-secreting cells by differentiation of embryonic or adult stem/progenitor cells has become the enthusiastic approach. In this study, progenitor cell lines from early (E6.0-7.5) mouse embryos were differentiated into insulin-producing cells that were characterized by RT-PCR, radioimmunoassay, glucose responsiveness profile, and animal transplantation experiments. These cells could be expanded for >7 months without loss of insulin-producing phenotype or compromise in cell proliferation rate. They expressed mRNA for both proinsulin-1 and -2, but not other islet hormones. Their insulin and C-peptide contents were almost equal (about 7 μg/$10^6$ cells), indicating predominant biosynthesis of insulin. Elevation of glucose levels in the medium from 2.8 to 7-15 mM evoked membrane depolarization, raised cytosolic free $Ca^{2+}$ levels and significantly increased insulin secretion in these cells. Similar to islet beta-cells, such glucose-induced insulin secretion was enhanced by cAMP-elevating agents, but suppressed by diazoxide (hyperpolarization), verapamil (blocking $Ca^{2+}$ entry), or inhibitory hormones (somatostatin or epinephrine). Transplantation of these cells into the kidney subcapsules in streptozotocin-induced diabetic mice was able to ameliorate the hyperglycemia. Such insulin-producing cells are also non-tumorgenic, as they did not induce teratomas when implanted in SCID mice. Our data have demonstrated that the insulin-producing cells derived from mouse embryo progenitor cells exhibit many key properties of primary beta-cells and can be propagated in vitro for a long period. Our findings provide strong evidence for the feasibility of generating functional insulin-secreting cells from human ES cells for type 1

diabetes therapy.

1426

**Morphological Peculiarities of Heart and Coronary Vessels Damage in Patients with Type 2 Diabetes Mellitus**

A. L. Davydov, L. Y. Baranova; Department of Endocrinology, Moscow State Medical Stomatological University, Moscow, Russian Federation

At present great attention is paid to investigation of cardiovascular pathology and vascular complications in type 2 diabetes patents. The aim of the work was to study morphological peculiarities of heart and coronary vessels damage in type 2 diabetes patients with late vascular complications. Materials and Methods: We studied endomiocardial bioptates of 7 patients with type 2 diabetes mellitus (DM) and without coronary atherosclerosis obtained during coronarography and ventriculography. After special treatment the sections of different thickness (thin and ultrathin) were examined under light and electron microscopes. Results: We revealed morphological peculiarities of myocardium and coronary vessels damage in type 2 DM patients without clinical signs of atherosclerosis and ischemic heart disease, in particular, pronounced fatty degeneration of cardiomiocites, accumulation of a - and b-glycogen in them, micromitochondriosis with marked polymorphism, miofibril lysis, sarcoplasmic reticulum defects, mitochondria destruction with crysts loss, apoptosis signs as well as arterioles and small and medium arteries changes, namely, pronounced edema of endotheliocytes, occurrence of fenestrated capillaries that are not normally characteristic of myocardium, and hypertrophy of unstriated muscle cells. These changes were responsible for decrease in useful heart work and disturbed coronary vessels function. Conclusion: The morphological peculiarities of myocardium and coronary arteries damage can be indicative of united pathogenetic mechanisms of development of such diseases as type 2DM, atherosclerosis, ischemic heart disease, and can be responsible for early development of failing heart in type 2 CD patients without coronary atherosclerosis.

1427

**Screening of Differentially Displayed Genes Correlative to Diabetes with and without Chinese Herb Astragalus Membranaceus Treatment**

H. F. Zhao, Y. Wu, S. L. Huang; Department of Biochemistry and Molecular Biology, Shanghai Second Medical University, Shanghai, China

The worst consequence of Diabetes mellitus (DM) at the terminal stage is irreversible renal fibrosis. Several clinical researches have indicated that Chinese herb Astragalus membranaceus (AM) has ability to defer the progression of DM. In this work, we studied the molecular mechanism of AM to defer renal fibrosis. A rat model of type 1 DM with two groups was built: diabetes with (DA group) and without (D group) AM treatment. The total RNA from renal tissues was used for gene chip analysis and some differentially displayed genes were confirmed by real-time PCR. Two hundred and one differentially displayed genes have been found in the chip with 4096 genes, in which 126 genes were up-regulated and 75 genes were down-regulated. One of expression down-regulated genes is *Latent Transforming Factor Binding Protein 1 (LTBP1)*. An interference plasmid of psilencer-shLTBP1 was constructed and transfected into HK2 cells. The changes of gene expression of *LTBP1, TGFβ1, collagenI and collagenIII* were detected by RT-PCR. In psilencer-shLTBP1 transfected HK2 cells, mRNA expression of *LTBP1, TGFβ1, collagenI* and *collagenIII* were down-regulated by 59%, 45%, 50% and 75% respectively. Our results suggest that *LTBP1* can regulate the expression of *TGFβ1* which improves the synthesis of collagen. LTBP1 itself may be a fibrosis associated factor. Our findings provide new insights into the molecular mechanism of AM to defer renal fibrosis via regulating *LTBP1* expression.

1428

**Constitutive Phosphorylation of GSK3-beta by High Extracellular Glucose Impairs CTGF Stimulated Migration in Human Cells**

F. Furlong, J. Crean, L. Thornton, R. O'Leary, M. Murphy, F. Martin; School of Biomolecular and Biomedical Research, Conway Institute, Dublin, Ireland

Diabetic nephropathy (DN) occurs in 30% of diabetic patients and is the leading cause of end stage renal disease. Increasing evidence indicates that hyperglycemia induced cytoskeletal disassembly or mechanical dysfunction contributes to mesangial cell pathology in DN. Our objective was to investigate mesangial cell migration in response to CTGF in glucose stress. Human mesangial cells (HMCs) cultured in high ambient glucose fail to migrate as normal in response to CTGF stimulation in a scratch wound assay. Failure of cell migration under these conditions was associated with failure of cell polarization events, e.g. redistribution of microtubules, in the direction of migration and non-responsiveness of the determining PKC-ζ-GSK3-β signaling axis. This latter intracellular signaling pathway, required for cell polarization, was constitutively activated under conditions of hyperglycemia. We showed that basal PKC-ζ and GSK3-β phosphorylation levels are selectively increased in HMCs exposed to high extracellular glucose for 48 h and that CTGF fails to further increase their levels. Acute exposure to high extracellular glucose (6 h) results in increased PKC-ζ and GSK3-β phosphorylation and this occurs downstream of p42/44 MAPK, PI 3-kinase and PKC-β activity. In normoglucose, CTGF induces HMC migration and the phosphorylation of PKC-ζ and GSK3-β, most likely through cdc-42 activation. However, after exposure to high glucose cdc-42 is also unresponsive to CTGF. Our data indicates that a tightly regulated and localized activation of signaling is essential for HMC migration in response to CTGF. The specific PKC-β inhibitor LY379196 suppressed the high glucose induced GSK3-β phosphorylation and restored CTGF-induced HMC migration in cultures exposed to high extracellular glucose. This hyperactivation of GSK3-β by PKC-β may establish a role for it in the progression of mesangial cell hyporesponsiveness in diabetic nephropathy progression.

1429

**Complete Proteomic Analysis of Purified Adiposomes from CHO K2 Cells**

R. Bartz,[1] J. K. Zehmer,[1] M. Zhu,[1] Y. Chen,[1] R. G. W. Anderson,[1] Y. Zhao,[2] P. Liu[1]; [1]Cell Biology, UT Southwestern Medical Center, Dallas, TX, [2]Biochemistry, UT Southwestern Medical Center, Dallas, TX

Adiposomes, or lipid droplets, are intracellular organelles with a neutral lipid core surrounded by a layer of phospholipid and protein. Recently, mass spectrometry has been used to identify the abundant proteins in the adiposome; however, potentially important low abundance proteins remain to be identified. Therefore, we used mass spectrometry to obtain a more complete picture of the proteins associated with highly purified CHO K2 adiposomes. Adiposome proteins were separated by one-dimensional gel electrophoresis and the gel cut into 34 pieces of equal size. The proteins in each piece were digested and the peptides were identified by mass spectrometry. This approach was supplemented by Western blot analyses of key proteins. We identified 122 proteins that include: 17 Rab GTPases; 14 proteins involved in membrane traffic; 14 cytoskeletal proteins; 16 proteins involved in lipid metabolism and 15 signaling proteins. Surprisingly, ARF1, another major membrane trafficking regulator, its

target protein membrin, and Arf GAP were among the proteins. We also found several proteins usually found in mitochondria, ER and peroxisomes, which is consistent with morphologic studies in yeast and metazoan cells showing an association of these organelles with lipid droplets. The abundance of proteins involved in intracellular trafficking and lipid synthesis support our hypothesis that adiposomes are organelles specialized not only for lipid storage but also intracellular lipid traffic and signal transduction.

1430

**High Content Screening of NPC1 Cells: Identification of Hit Compounds that Reduces Cholesterol Accumulation in Cultured Cells**

N. H. Pipalia, A. Y. Huang, H. Ralph, M. Rujoi, F. R. Maxfield; Biochemistry, Weill Medical College of Cornell University, New York, NY

High content screening of NPC1 cells: Identification of hit compounds that reduces cholesterol accumulation in cultured cells Niemann-Pick disease type C1 (NPC1) is an autosomal recessive genetic disorder that causes an abnormal accumulation of cholesterol and other lipids in many cell types. Despite several attempts to develop treatments for NPC1 disease currently there is no effective therapy to correct this disorder. In order to identify chemicals that would correct the NPC1 phenotype in tissue culture cells, previously we developed a robust high content microscopy based assay to screen a library of combinatorially synthesized compounds (ChemDiv, Inc.). We chose CHO cells with NPC1 defects as our model system and exploited fluorescent property of cholesterol-binding detergent filipin for automated microscopy based high throughput screening. Our primary screen of 14,956 chemicals had identified 14 compounds that reproducibly reduced filipin staining in cells at 10 μM. A secondary screen of an additional 3962 structurally similar compounds to the primary hits identified an additional 6 compounds that are non-toxic and effective at sub-μM concentrations in reducing free cholesterol content as observed by reduction in filipin intensity. The screening results were validated by an alternative biochemical determination of free cholesterol content by gas chromatography. Preliminary results indicate that some of these compounds are also effective in reducing cholesterol accumulation in human fibroblasts obtained from NPC1 patients. We are now screening the secondary library on two human fibroblast lines obtained from NPC1 patients. The molecular mechanism and structure activity relationship for these classes of compounds is also being evaluated. Supported by the Ara Parseghian Medical Research Foundation.

1431

**Study of Cholesterol Pathways Affected by Small Molecules that Partially Revert Niemann-Pick Type C1 Phenotype in Cultured Cells**

M. Rujoi, N. H. Pipalia, A. Y. Huang, H. Ralph, F. R. Maxfield; Biochemistry, Weill Medical College of Cornell University, New York, NY

Niemann-Pick type C1 disease (NPC1) is a fatal neurovisceral multi-lipid-storage disorder. Its hallmark is the defect in cholesterol efflux from lysosomal storage organelles (LSOs). Effective therapeutic treatments are not available. Based on the cholesterol-binding fluorophore, filipin, our lab developed an automated fluorescence microscopy screening method to identify chemicals that correct NPC1 phenotype in cultured cells. From a screen of 14,956 compounds and a follow-up of additional 3,962 we identified 2 sets (S1 and S2) of 14 and 6 hit compounds, respectively that reduce filipin staining of LSOs. Moreover, compounds from S2 decrease the overall cellular cholesterol levels, as confirmed by gas chromatography analysis. The impact of selected compounds on the major intracellular cholesterol pathways in NPC1 cultured cells was analyzed. Cholesterol export from LSOs and transport to extracellular acceptors was monitored before and after compound-treatment using [$^{3}$H]-cholesterol. The majority of compounds (10 chemicals from S1 and 5 from S2) increased cholesterol efflux. A second key pathway is the transport to the ER, where cholesterol gets esterified by acyl-coenzyme A: cholesterol acyltransferase (ACAT). The cholesterol ester formed by ACAT is incorporated in lipid droplets and can then be hydrolyzed by neutral cholesteryl ester hydrolase (NCEH). Incorporation of [$^{14}$C]-oleate into cholesteryl-[$^{14}$C]-oleate was used as a measure of cholesterol esterification by ACAT. Among the 20 selected compounds only one promotes ACAT-mediated esterification. However, quantification by gas chromatography of esterified cholesterol revealed increased ester levels upon treatment with 7 compounds from S1 and 5 from S2. Inhibition of NCEH upon compounds addition could explain the increase in cholesterol ester levels. In conclusion, cholesterol extracellular efflux and/or esterification are the main mechanisms affected by selected compounds to partially correct NPC defect in cultured cells.

1432

**Defective Endocytic Pathways in Lipid Storage Disorders**

S. Muro,[1,2] V. Muzykantov,[1,2] E. Schuchman[3]; [1]Institute for Environmental Medicine, University of Pennsylvania Medical School, Philadelphia, PA, [2]Department of Pharmacology, University of Pennsylvania Medical School, Philadelphia, PA, [3]Department of Human Genetics, Mount Sinai School of Medicine, New York, NY

Lipid storage caused by inherited deficiency of lysosomal hydrolases, cofactors or transporters, impairs intracellular lipid trafficking. Poor uptake of recombinant hydrolases by storage cells suggests that internalization of extracellular ligands may also be impaired. To evaluate whether endocytosis is affected in these diseases, we used fluorescence microscopy to test dextran pinocytosis, clathrin-mediated uptake of transferrin, and caveoli-mediated uptake of cholera-toxin by skin fibroblasts from Gaucher, Fabry, type B and C Niemann-Pick (NPB, NPC) patients, characterized by deficiency of glucocerebrosidase, α-galactosidase-A, acid sphingomyelinase, and NPC1 transporter, respectively. Normal cells internalized 120±14 dextran-positive vesicles (1h-37°C). MDC, filipin, and amiloride, which inhibit uptake by clathrin pits, caveoli, and macropinocytosis, decreased dextran internalization (52±8%, 68±10%, 63±10%). A cocktail of the three drugs abolished uptake (7±4%), indicating that the three pathways contribute to fluid-phase endocytosis. Dextran uptake was reduced in all storage cells (<75±6% of normal cells). MDC did not further inhibit dextran uptake (>92±13% of untreated cells). Inhibition by filipin was less marked for NPB and NPC (>84±11%), and inhibition by amiloride was decreased in NPB (81±11%) and increased in NPC (34±6%). Dextran uptake in EGF-treated cells, inducing macropinocytosis, was decreased in NPB and increased in NPC (80±10% and 155±12% of normal cells). Internalization of cholera-toxin pre-bound to the cell membrane was decreased in NPB and NPC (<63±6% of normal cells). Uptake of pre-bound transferrin was affected in all storage cells (<48±3% of normal cells). These results indicate that: 1-clathrin-mediated endocytosis is affected in Gaucher, Fabry, NPB and NPC; 2-uptake via caveoli is decreased in NPB and NPC; and 3-macropinocytosis is slightly decreased in NPB and increased in NPC. Altered endocytosis may represent a common mechanism contributing to cellular dysfunction in storage disorders, and may add to suboptimal enzyme delivery by therapeutic strategies based on deficient receptors.

1433

**IFN-gamma-inducible Protein (IP-10) Produced By Osteoclast Precursors Mediates Rheumatoid Arthritis**

H. Kwak, D. Yang, H. Ha, H. Kim, J. Lee, H. Kim, H. Kim, Z. Lee; Cell and Developmental Biology, Seoul National University School of Dentistry, Seoul, Republic of Korea

IFN-γ-inducible protein (IP-10), a CXC chemokine important in the activation of T cells, is induced by IFN-γ in a variety of cell types and plays a role in the infiltration of T cells to sites of inflammation. However, the roles of IP-10 in rheumatoid arthritis are mainly unknown. Here we show that the expression of IP-10 was increased in RANKL stimulated osteoclast precursors but not in mature osteoclasts. RANKL induces IP-10 expression through a STAT1-dependnet pathway in osteoclast precursors. IP-10 stimulates the expression of RANKL and TNF-α in $CD4^+$ T cells and induces osteoclastogenesis. We examined the role of IP-10 in a mouse model of collagen-induced arthritis (CIA). ELISA analysis revealed that the expression of IP-10, RANKL, and TNF-α increased in serum of CIA mice. Treatment with the neutralizing anti-IP-10 antibody significantly inhibited infiltration of $CD4^+$ T cells and macrophages into the synovium and attenuated bone destruction in the inflamed joints of CIA mice. Furthermore, the levels of RANKL and TNF-α were inhibited by anti-IP-10 antibody in CIA mice. Also, bone erosion in the knee joint was observed in mice infected with an IP-10-containing retrovirus. Taken together, our findings suggest that IP-10 plays a critical role in the infiltration of $CD4^+$ T cells and macrophage into inflamed joints and causes osteoclastogenesis through production of RANKL and TNF-α. Our result provides the first evidence that IP-10 contributes to the recruitment of inflammatory cells into joint and is involved in rheumatoid arthritis.

1434

**Rhodopsin Resists Oxidative Modification During Light- and Metabolically-Induced Retinal Degeneration**

S. J. Fliesler,[1] M. J. Richards,[2] B. J. Kapphahn,[3] D. A. Ferrington[3]; [1]Ophthalmology and Pharmacol. Physiol. Sci., Saint Louis University, St. Louis, MO, [2]Ophthalmology, Saint Louis University, St. Louis, MO, [3]Ophthalmology, Univ. of Minnesota, Minneapolis, MN

Oxidation of proteins has been implicated in various neurodegenerative processes. This includes covalent modification with lipid-derived adducts, such as 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE), and carboxyethylpyrrole (CEP), as well as nitrosylation of tyrosine (NT). Retinal degeneration is exacerbated by exposure to intense constant light ("retinal light damage"). Lipid hydroperoxide accumulation correlates positively with retinal degeneration in both albino rats exposed to light-damage conditions (see Organisciak & Winkler, *Prog. Ret. Eye Res.* 13:1, 1994), and in a rat model of Smith-Lemli-Opitz Syndrome (SLOS; see Fliesler et al., *Arch. Ophthalmol.* 122:1190, 2004). Here, we examined oxidative modification of rhodopsin, the retinal rod visual pigment and predominant outer segment (OS) membrane protein, in these two retinal degeneration models. Pairs of animals were exposed for 24 h to intense green light (1700 lux, 490-580 nm), while the unexposed cohort was kept in darkness. OS membranes were prepared from pairs of retinas for each condition; in parallel, one eye of each animal was taken for morphological analysis. Proteins were resolved by SDS-PAGE, with and without pre-boiling: one gel from each group was silver-stained (SS), while companion gels were blotted and probed with polyclonal antibodies to opsin, HNE, HHE, CEP, or NT. Rhodopsin was not immunopositive to any of these antibodies. Upon boiling, it oligomerized and shifted to the stacker-resolving gel interface, exposing a set of immunopositive bands in the $M_r$ 31-45 kDa region (previously including rhodopsin). We conclude that rhodopsin resists oxidative modifications induced by either intense light or under conditions that mimic SLOS, while other OS-resident proteins do become oxidized. Thus, oxidative damage to opsin is not a primary event in the etiology of these retinal degenerations. [EY07361, EY14176, RPB]

1435

**TRIM37 Is an Ubiquitin E3 Ligase That Interacts with Aryl Sulphotransferase SULT1A2**

J. P. Kallijarvi, R. H. Hamalainen, A. E. Lehesjoki; Dept. Medical Genetics, University of Helsinki and Folkhalsan Institute of Genetics, Helsinki, Finland

Mulibrey nanism (muscle-liver-brain-eye nanism) is a rare growth disorder syndrome of prenatal onset. Mutations in the *TRIM37* gene underlie mulibrey nanism. To date, 11 mulibrey nanism-associated mutations, of which nine are truncating and two are missense mutations, have been described. The pathogenetic mechanisms of mulibrey nanism are unknown. *TRIM37* encodes a member of the TRIM (tripartite motif, RING-B-Box-Coiled-Coil) protein subfamily of zinc-finger proteins, many of which possess ubiquitin ligase activity. We have shown that TRIM37 is subject to RING domain-dependent autoubiquitination, suggesting that it functions as an ubiquitin ligase. A patient mutation causing a Leu76Pro amino acids change, located between the RING and the first B-box domain of TRIM37, abolishes autoubiquitination suggesting that ubiquitin ligase activity is central to the pathogenesis of mulibrey nanism. Here, we used yeast two-hybrid screening to identify proteins that interact with TRIM37. In a screen with a TRIM domain bait we identified a clone containing a putative splice variant of *hast4/SULT1A2* cDNA. GST pull-down experiments confirmed that both the alternative isoform encoded by the yeast clone and the full-length SULT1A2 protein, but not the related enzymes SULT1A3 and SULT1C1, bind to the TRIM domain of TRIM37. SULT1A2 showed a homogenous cytosolic localization when expressed in COS-1 cells. When co-expressed with TRIM37 SULT1A2 localized to cytosolic aggregates. Moreover, co-expression of SULT1A2 and TRIM37 in COS-1 cells resulted in polyubiquitination of SULT1A2. Sulfonation is an important step in the metabolism of many drugs, xenobiotics, neurotransmitters, and steroid hormones. Mulibrey nanism patients display multiple endocrine defects, including hypogonadism, failure of sexual maturation and adrenal hypoplasia. The identification of SULT1A2 as an interaction partner for TRIM37 raises the possibility that dysregulated metabolism of steroid hormones and/or neurotransmitters could play a role in the pathogenesis of mulibrey nanism.

1436

**Unraveling the Cell Biology of 3-Methylglutaconic Aciduria**

M. Huizing,[1] L. Ly,[1] H. Dorward,[1] B. Feldman,[1] Y. Anikster,[2] W. A. Gahl[1]; [1]NHGRI, NIH, Bethesda, MD, [2]Safra Children Hospital, Sheba Medical Center, Tel-Hashomer, Israel

3-Methylglutaconic aciduria (3MGA) comprises a group of urinary organic acid abnormalities, which can result from mitochondrial defects (leucine degradation) or peroxisomal defects (mevalonate shunting). Types I and II 3MGA are caused by defects in mitochondrial proteins, and Type III 3MGA results from abnormalities of OPA3, a protein of unknown function. In addition, a large group of patients exist with unexplained 3MGA (Type IV 3MGA). Since OPA3 contains an N-terminal mitochondrial leader sequence and a C-terminal peroxisomal targeting signal, we studied OPA3 function to gain more insights into 3MGA metabolism. The *OPA3* gene is known to consist of two exons coding for 179 amino

acids. However, we identified a novel *OPA3* transcript consisting of the common exon 1 spliced directly to a 3rd exon (ex 1-3). This transcript is conserved in other species, and exon 3 closely resembles exon 2, suggesting a common evolutionary origin. Molecular analysis of 11 patients with type IV 3MGA did not reveal exon 3 mutations. Normal fibroblasts were electroporated with Green Fluorescent Protein-tagged OPA3 fusion proteins (ex 1-2 and ex 1-3 transcripts) with or without mutated mitochondrial and/or peroxisomal targeting signals. Confocal microscopy clearly demonstrated a mitochondrial localization for both transcripts (ex 1-2 and ex 1-3). However, only the ex 1-2 transcript also localized to peroxisomes. This rare dual localization could help further elucidate the biochemical function of OPA3. To further explore OPA3 function, we created zebrafish models using antisense morpholinos. The mutant fish showed unique features, including small eyes with severely affected optic nerves, delayed development, kinked tails and spastic movements - all resembling human Type III 3MGA symptoms. These zebrafish models promise to further reveal the biochemical function of OPA3 and assist in the development of possible therapies for this group of disorders.

1437

**Fucose Mutarotase, a Novel Component in Fucose Utilization via the Salvage Pathway**

D. Park, K. Kim, D. Choi, C. Park; Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea

Monosaccharides exist as a cyclical pair of isomers, the α- and β- anomers, that differ only in the configuration of the anomeric carbon atom. L-fucose is a monosaccharide normally present in mammals and is utilized presumably for glycosylation. The first step in intracellular fucose utilization through the salvage pathway is known to be a phosphorylation by fucokinase. We discovered a novel component in fucose utilization, which involves the interconversion between the α- and β- anomers of fucose. The mouse and human genes encoding fucose mutarotases belong to the FucU family of proteins, whose prokaryotic homologs have been characterized recently (Ryu *et al.*, 2004, JBC, 279: 25544-8). Using the saturation difference NMR analysis, we were able to demonstrate that the mammalian homologs have similar mutarotase activities. When we overproduced the mutarotase by generating a stable cell line from HepG2, intracellular accumulation of lipid as well as a change in cell morphology was observed. We speculate that this may occur due to a facilitated incorporation of fucose through the salvage pathway, eventually leading to an enhanced glycosylation of various cellular proteins.

1438

**Evaluation of the Glycosylation Status of Alpha-dystroglycan in Hereditary Inclusion Body Myopathy (HIBM)**

P. J. Savelkoul,[1] S. Sparks,[1] G. Rakocevic,[2] R. Klootwijk,[1] C. Ciccone,[1] M. Dalakas,[2] D. Krasnewich,[1] W. A. Gahl,[1] M. Huizing[1]; [1]Section on Human Biochemical Genetics, MGB, NHGRI, NIH, Bethesda, MD, [2]Neuromuscular Disease Section, NINDS, NIH, Bethesda, MD

Hereditary Inclusion Body Myopathy (HIBM) is an adult onset autosomal recessive neuromuscular disorder characterized by slowly progressive myopathic weakness and atrophy. HIBM is caused by mutations in UDP-GlcNAc 2-epimerase (GNE)/N-acetylmannosamine kinase (MNK), the bi-functional and rate-limiting enzyme in sialic acid biosynthesis. We developed individual GNE and MNK enzymatic assays and determined reduced activities in cultured fibroblasts of patients with HIBM harboring missense mutations in either or both GNE and MNK enzymatic domains. To assess the effects of individual mutations on enzyme activity, normal and mutated GNE/MNK enzymatic domains were synthesized in a cell-free *in vitro* transcription-translation system and subjected to the GNE and MNK enzymatic assays. This revealed that mutations in one enzymatic domain affected not only that domain's enzyme activity but also the activity of the other domain. This loss of enzyme activity impairs sialic acid production, which may interfere with proper sialylation of glycoconjugates. We investigated how this would lead to muscle pathology. First, we demonstrated normal iso-electric focusing (IEF) patterns of transferrin in HIBM patients' serum, suggesting normal N-linked glycosylation. Next, we performed immunohistochemistry on HIBM muscle employing antibodies against components of the dystrophin-glycoprotein complex. Beta-dystroglycan and laminin-alpha 2 showed normal patterns, but antibodies recognizing O-linked glycan epitopes of alpha-dystroglycan (α-DG) showed reduced staining. α-DG contains both O-GalNAc and O-mannose linked glycans; the latter are rare in mammals. Finally, we showed normal IEF patterns of apolipoprotein C-III, which contains only O-GalNAc linked glycans, suggesting that O-GalNAc glycosylation is unaffected in HIBM. These findings suggest a defect in O-mannosylation of α-DG in HIBM. Understanding the function and regulation of the O-linked mannose pathway is essential for developing diagnostic tests and therapies for HIBM and other muscular dystrophies with similar pathology.

1439

**Tissue Specificity and Evolutionary Conservation of Vps33 Homologues**

P. Gissen,[1] C. A. Johnson,[1] L. D. Hurst,[2] A. J. Doherty,[3] E. R. Maher[1]; [1]Section of Medical and Molecular Genetics, University of Birmingham, Birmingham, United Kingdom, [2]Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom, [3]UK Genome Damage and Stability Centre, University of Sussex, East Sussex, United Kingdom

Mutations in *VPS33B* cause ARC syndrome, a lethal multi-system disease which affects infants and cause abnormalities of the liver, kidneys, central nervous system and platelets.VPS33B protein is a homologue of yeast class C vacuolar protein sorting (Vps33p) protein, involved in biogenesis and function of vacuoles. Two Vps33 homologues (A and B) are present in most organisms. There are no mouse *VPS33B* knockouts to model ARC, but inactivation of mouse *Vps33a* causes *buff* phenotype, characterized by pigment defects and platelet abnormalities. **Objectives.** Identify functional characteristics of the A and B homologues of Vps33p in different organisms. **Methods.** After performing homology searches, multiple sequence alignment for the vps33p protein homologues was performed using ClustalW. Two phylogenetic trees were then created using MrBayes and also Tree Puzzle, a quartet puzzling maximum likelihood method. Putative functional motifs were modeled using Rasmol. Evolutionary conserved regions in VPS33B and VPS33A were identified by calculating Ka/Ks ratio. Labeled wild-type and truncated constructs of Vps33p homologues were transfected into human renal cells and also mouse melanocytes and the effect of clustering on lysosome related organelles was assessed by immunofluorescence. **Results.** Two homologues of yeast vps33p were identified in the worm, fly, zebrafish, rodent and human genomes. A novel *Drosophila* orthologue of VPS33B was identified, whereas *car* gene product Carnation was found to be an orthologue of VPS33A rather than VPS33B. Conserved functional domains within VPS33B were found and their deletion impaired the clustering effect of the expressed protein. VPS33B induced significantly more clustering of late endosomes and lysosomes than VPS33A in renal cells whilst VPS33A was more efficient at inducing clustering of melanosomes in melanoma cells. This is the first documented evidence of organelle and tissue specificity of the clustering effect.

1440

**Constitutive Clathrin-Mediated Endocytosis of ATP-Sensitive Potassium Channels: the Role of Tyrosine-Based Signals**

A. J. Smith,[1] J. Mankouri,[1] T. K. Taneja,[1] S. Ponnambalam,[2] A. Sivaprasadarao[1]; [1]School of Biomedical Sciences, University of Leeds, Leeds, West Yorkshire, United Kingdom, [2]School of Biochemistry and Microbiology, University of Leeds, Leeds, West Yorkshire, United Kingdom

ATP-sensitive potassium ($K_{ATP}$) channels are involved in numerous physiological processes including the regulation of insulin secretion and the protection of neuronal and cardiovascular cells during periods of metabolic stress. Pancreatic $K_{ATP}$ channels are octomers composed of 4 subunits each of Kir6.2 and SUR1. Because their level of activity is determined not only by the open probability, but also by the density of the channels at the cell surface, we investigated if these channels can undergo endocytosis. For this, we tagged the channels with an extracellular HA epitope in the Kir6.2 subunit, expressed in mammalian cell lines, and examined the mechanism of internalisation using immunofluorescence/confocal microscopy and biochemical approaches. Site-directed mutagenesis was used to alter the sequence of putative motifs required for endocytosis. Our data demonstrate that $K_{ATP}$ channels undergo rapid clathrin-dependent internalisation from the cell surface with subsequent trafficking to perinuclear compartments that are positive for TGN46 and Mannose-6-Phosphate Receptor. Co-localisation with the transferrin receptor positive recycling compartment was found to be limited. Motifs involved in endocytosis seem to be located exclusively in the C-terminal tail of the Kir6.2 subunit. Whilst mutation of the $^{355}$LL$^{356}$ motif has no significant effect on internalisation, mutation of either of the two tyrosine-based motifs ($^{258}$YHVI$^{261}$ and $^{330}$YSKF$^{333}$) inhibited internalisation significantly. Mutation of both tyrosines, however, was required to completely prevent endocytosis, suggesting that multiple signals control internalisation, and hence the surface density of the channels. These findings may have implications for insulin secretion and other $K_{ATP}$-dependent protective mechanisms during ischemia of the heart and the brain. Funded by the MRC-UK.

1441

**Characterization of a Hyaluronidase 1 Deficient Mouse Model**

D. C. Martin, V. Atmuri, R. J. Hemming, B. L. Triggs-Raine; Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada

Hyaluronidases are endoglycosidases that degrade hyaluronan (HA), an abundant component of the extracellular matrix surrounding all vertebrate cells. HA is especially abundant in loose connective tissues and in processes that require rapid proliferation and migration. While six hyaluronidase genes are known in humans, their roles in HA degradation have not been elucidated. A deficiency of one of these enzymes, Hyaluronidase 1 (HYAL1), causes a lysosomal storage disease with a mild clinical phenotype. We obtained an uncharacterized Hyal1 deficient mouse model (Stern et al., *Matrix Biol.* 20(8):499). Our objective is to examine the Hyal1 contribution to HA degradation and the pathology associated with its deficiency. Wild-type (+/+), heterozygous (+/-) and deficient (-/-) animals were generated. We have used Southern blotting and sequencing of PCR products generated from -/- genomic DNA to demonstrate that the neomycin resistance (Neo) cassette is inserted in exon 2 of *Hyal1*. This resulted in a deletion of 750 bp of coding sequence including the predicted active site of the protein. Deficient animals reveal no wild-type *Hyal1* transcript by RT-PCR analysis of liver mRNA. Northern analysis of surrounding genes shows no alteration in their levels of expression due to the incorporation of the Neo cassette. Deficient animals contain no detectable Hyal1 protein or serum hyaluronidase activity by immunoblot and zymographic analysis respectively. Preliminary results of heterozygous breedings suggest an altered male sex ratio. Instead of a 1:2:1 (+/+: +/-: -/-) genotype ratio, a 1:2:2 (n= 45) ratio is observed. Animals display no gross phenotypic abnormalities and are healthy up to 1 year of age. Histological examination of tissues to examine morphology and substrate accumulation is in progress. Our data indicates that this represents a model of complete Hyal1 deficiency and there are no gross morphological changes due to HA accumulation.

1442

**Trafficking of Human Lysosomal Sialidase (neu1) to the Cell Surface**

S. Pattison,[1] J. P. King,[1] H. Kufaishi,[1] D. Aboutouk,[1] R. Truant,[2] B. Trigatti,[2] S. A. Igdoura[1,3]; [1]Biology, McMaster University, Hamilton, ON, Canada, [2]Biochemistry, McMaster University, Hamilton, ON, Canada, [3]Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada

Lysosomal sialidase hydrolyzes sialic acid from glycolipids and glycoproteins. The interaction between cathepsin A, β-galactosidase, and sialidase is required for sialidase localization and activity within lysosomes. The role of cathepsin A, is closely tied to targeting of sialidase and catalytic activation. Trafficking of human lysosomal sialidase has been reported to be mediated by binding to immature cathepsin A (CA), and utilizing the CA mannose 6-phosphate receptor signal for transport from the Golgi to the lysosome directly. However, other studies suggested that T-cells have immature complexes of cathepsin A-sialidase at their cell surface, postulating the possibility of an indirect trafficking pathway from the Golgi to the surface membrane to the lysosome. In this study, we created truncated forms of the sialidase and examined their intercellular localization and their association with PPCA. The truncations represented progressively shorter carboxyl termini (p.N261X, p. G321X and p.S355X). Using immunofluorescence and co-localization of the truncated sialidase mutants with Golgi, lysosomal and endosomal markers, we determined the intercellular localization of each truncation mutant. Using a sialidase-monomeric red fluorescent protein fusion construct (Sial-mRFP) and live real time microscopy, we investigated the trafficking pathway of lysosomal sialidase in primary sialidosis fibroblasts and HeLa cells. We combined the sial-mRFP with markers for Golgi and endosomes and determined that Sial-mRFP co-localizes with Golgi elements but not with endosomes. Additionally, treatment with Brefeldin A for 15 minutes revealed a transient appearance at the cell membrane followed by the presence in lysosome-like punctate structures which were not endosomes. We conclude that sialidase follows an indirect pathway to the lysosome via the cell surface; however, that routing mechanism may not require the putative YXXϕ present on its carboxyl terminal.

## Other Diseases I (1443-1464)

1443

**The mTOR Pathway is Regulated by Polycystin-1 and Pharmacological Inhibition of mTOR Significantly Improves the Renal Cystic Phenotype in Polycystic Kidney Disease**

J. M. Shillingford,[1] N. S. Murcia,[2] S. Low,[1] R. Hedgepeth,[3] N. Brown,[2] C. A. Flask,[4] A. C. Novick,[3] D. A. Goldfarb,[3] A. Kramer-Zucker,[5] G. Walz,[5] K. B. Pinotek,[6] G. G. Germino,[6] T. Weimbs[1]; [1]Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA, [2]Department of Pediatrics, Case Western Reserve University, Cleveland, OH, [3]Glickman Urological Institute, The Cleveland Clinic,

Cleveland, OH, [4]Departments of Radiology and Biomedical Engineering, Case Western Reserve University, Cleveland, OH, [5]Department of Medicine, Nephrology, University Hospital Freiburg, Freiburg, Germany, [6]Department of Medicine, Johns Hopkins University, School of Medicine, Baltimore, MD

The objective of this work was to investigate the contribution of the mammalian target of rapamycin (mTOR) pathway as it relates to the development of renal cysts in polycystic kidney disease (PKD). Herein we show that mTOR and a downstream effector, S6 kinase, are specifically phosphorylated in cyst-lining epithelial cells of renal samples derived from human autosomal dominant PKD (ADPKD) patients and mouse models of PKD but not normal renal samples. Furthermore, using a renal (MDCK) cell culture model engineered to express selective portions of the polycystin-1 (PC1) cytoplasmic tail we have shown that the N-terminal half of the PC1 tail co-localizes with mTOR and tuberin and, by co-immunoprecipitation, directly binds to tuberin. We hypothesized that if the mTOR pathway contributes to renal cyst formation then inhibition of mTOR activity would slow disease progression. To test this hypothesis we treated two genetic mouse PKD models with rapamycin, a specific mTOR inhibitor. These studies revealed that rapamycin treatment significantly improved the renal cystic phenotype as assessed by decreased serum blood urea nitrogen (BUN) levels, kidney weight and cystic index. To demonstrate relevance to human ADPKD we took advantage of the fact that some ADPKD patients retain at least one affected kidney and receive rapamycin treatment in the form of immunosuppressant therapy following renal transplantation. Using predetermined criteria for patient selection and volume analysis of CT scans we documented a significant reduction in ADPKD kidney volume in rapamycin- vs. non-rapamycin-treated patients. In conclusion, we have demonstrated that the mTOR pathway is abnormally and specifically activated in cyst-lining epithelial cells in ADPKD and present data that rapamycin treatment may represent a means of therapeutic intervention in this disease.

1444

### Absence of Flow-induced $Ca^{2+}$ Signaling in Human Autosomal Dominant Polycystic Kidney Disease Cyst Cells

C. Xu,[1] R. L. Bacallao,[2] S. Rosetti,[3] P. C. Harris,[3] A. Wandinger-Ness,[4] S. L. Alper[1]; [1]Medicine, Beth Israel Deaconess Med. Ctr., Harvard Med. School, Boston, MA, [2]Medicine, Indiana Univ. School Med, Indianapolis, IN, [3]Medicine, Mayo Medical School, Rochester, MN, [4]Medicine, University of New Mexico Health Sci. Ctr., Albuquerque, NM

Aberrant function of the primary apical cilium of renal epithelial cells is thought to underlie the pathogenesis of autosomal dominant polycystic kidney disease (ADPKD). The PKD1 gene product polycystin-1 (PC1) and the PKD2 gene product polycystin-2 (PC2) co-localize in cilia of mouse renal epithelial cells, where they are believed to contribute to flow-sensitive mechanosensing. We have found that acetylated α-tubulin-positive cilia of confluent normal human kidney epithelial cells in primary culture contain both PC1 and PC2. However, PC1 is only rarely detected in PC2-positive cilia of cyst cells obtained from an ADPKD patient with a novel heterozygous germline deletion of one amino acid in exon 18 of the PKD1 gene. Confluent normal kidney cells respond to graded increases in laminar shear stress with graded, transient increases in $[Ca^{2+}]_i$ dependent on extracellular $Ca^{2+}$, but laminar flow fails to elicit elevation of $[Ca^{2+}]_i$ in ADPKD cyst cells. Flow-induced $Ca^{2+}$ signaling is blocked by the mechanosensitive channel (MSC) inhibitor GsMTx-IV, by ryanodine and by 2-APB, but not by the phospholipase C inhibitor U73122. Expression at the normal cell surface and primary cilium of a transmembrane fusion protein encoding the C-terminal portion of the PKD1 cytoplasmic tail, PKD1(115-226) partially inhibited flow-induced $[Ca^{2+}]_i$ increase. These data support the hypothesis that normal function of PC1 in the primary cilium in human kidney epithelial cells contributes to mechanosensitive $Ca^{2+}$ influx. (Supported by NIH DK57662 and DK69049).

1445

### Mutant α-Actinin-4 Causes Defects in Cytoskeletal Dynamics and Cell Motility

J. R. Michaud, C. R. J. Kennedy; Ottawa Health Research Institute, Kidney Research Centre, Ottawa, ON, Canada

Mutations in the *ACTN4* gene, encoding the actin filament crosslinking protein α-actinin-4, are associated with a familial form of focal segmental glomerulosclerosis (FSGS). α-Actinin-4 is highly expressed in the foot processes of glomerular podocytes and plays a key role in maintaining the cell's arborized morphology. Though FSGS-associated mutant α-actinin-4 exhibits increased affinity for actin, the downstream effects of this aberrant binding upon cellular dynamics and morphology remain unclear. When overexpressed in mouse podocytes, wildtype α-actinin-4 localized to cortical actin and focal adhesions, with some expression along actin stress fibers. Mutant α-actinin-4 associated predominantly with stress fibers and focal adhesions however, it was excluded from cortical actin. Wildtype and mutant α-actinin-4-expressing cells adhered equally to an extracellular matrix (collagen I) as determined by microplate adhesion assay. When replated onto collagen I-coated coverslips, wildtype but not mutant α-actinin-4-expressing cells spread efficiently. In these newly adherent cells, wildtype α-actinin-4 was localized to the cell membrane, while mutant α-actinin-4 remained condensed within the rounded cell body. Mutant α-actinin-4 also reduced cell motility by 50% as determined by haptotaxis assay. Lastly, wildtype α-actinin-4 favored the formation of long and narrow projections, reminiscent of podocyte foot processes observed *in vivo*. Expression of wildtype α-actinin-4 increased the mean number of processes (5.2 /cell vs. 3.9 /cell for control), while cells expressing mutant α-actinin-4 had fewer processes (2.6 /cell). These data suggest that aberrant sequestering of mutant α-actinin-4 results in significant defects in cell spreading and motility. Furthermore, mutant α-actinin-4 reduces the number of podocyte processes, and may thereby contribute to foot process effacement observed in *ACTN4*-associated FSGS.

1446

### NIMA-Related Kinases Defective in Murine Models of Polycystic Kidney Diseases Localize to Primary Cilia and Centrosomes

M. R. Mahjoub, M. L. Trapp, L. M. Quarmby; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

Polycystic kidney disease (PKD) is one of the most common genetic diseases, affecting 12.5 million people worldwide. Several proteins involved in the polycystic pathology are found at primary cilia, basal bodies, and cell-cell junctions. A key feature of the etiology of cyst formation is aberrant cell proliferation, presumably a consequence of dysfunctional regulation of the cell cycle by basal bodies/centrioles and primary cilia [reviewed by Pazour, 2004, J. Am. Soc. Nephrol. 15, 2528-2536]. The NIMA-related kinases (Neks) Nek1 and Nek8 carry the causal mutations of two of the eight established mouse models of PKD [reviewed by Guay-Woodford, 2003, Am. J. Physiol. Renal Physiol. 285, 1034-1049] and members of this kinase family have cell cycle roles [O'Connell et al., 2003, Trends Cell Biol. 13, 221-228]. Here, we report the intracellular localization of endogenous Nek1 and Nek8 in mouse kidney epithelial cell line. Nek1 localizes to centrosomes and Nek8 to the proximal region of the primary cilium. During mitosis, Nek1 remains associated with the centrosome of the mitotic spindle, whereas Nek8 is not observed in dividing

cells. Knockdown of Nek8 results in loss of staining at the cilia, while not affecting ciliary assembly. Taken together with the phenotypes of the mutant mice, our data suggest that Nek1 and Nek8 provide a direct link between cilia, centrosomes, and cell cycle regulation.

1447

**The Injury of Hepatocytes of HBx Transgenic Mice and the Protective Role of Selenium**

Y. Tu,[1] Z. Qi,[1] J. Chen,[2] P. Yang,[1] Y. Pan,[1] S. Yu[3]; [1]Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China, [2]Department of Pathology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China, [3]Department of Biochemistry and Molecular Biology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China

HBx gene product is a pathogenic factor related to human hepatitis B virus (HBV) infection. In order to obtain an animal model for mimicing the chronic injury of hepatocytes caused by HBV and using it to find a protective treatment against the injury of the cells, the HBx transgenic mice (TG) were generated. The TG with or without $Na_2SeO_3$ diet and wild type mice (WT) were maintained and samples were collected for Southern and Northern blot hybridization analysis and pathological examination. The results indicated that the hepatocytes of 2- month old TG were normal. However, 50% of the 8-month old TG showed slight inflammatory cell infiltration and all of the 16-month old TG had serious inflammatory changes of the cells (eg. scattered focal necrosis with distinct inflammatory cell infiltration etc.). Moreover, 50% of 16-month old TG were found experiencing possible precancerous changes. After treatment with 1ppm sodium selenium, the hepatocytes of TG were as normal as controls although those mice were showing an integration and expression of HBx gene in hepatocytes. In summary, the expression of HBx gene in TG can induce a chronic injury of hepatocytes thus the TG as a model have been successfully developed. The selenium can act as a protective reagent against the cellular injury caused by HBx gene expression.

1448

**Inhibition of Ethanol-induced Cytotoxicity in Cultured Hepatocytes by RNAi Silencing of Par-4**

J. Xie, Q. Guo; Department of Physiology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK

The precise mechanisms underlying alcoholic liver disease are not completely understood, although several lines of experimental evidence suggest exposure of hepatocytes to ethanol may induce apoptosis via increased oxidative stress and induction of pro-apoptotic machinery. Par-4 (prostate apoptosis response-4) is a pro-apoptotic protein ubiquitously expressed in many tissues, including the liver. Par-4 contains both a death domain and a leucine zipper domain and its expression is up-regulated in many types of cells induced to undergo apoptosis. Par-4 functions early in apoptotic machinery, upstream of caspase activation and mitochondrial dysfunction. We now report that Par-4 is expressed in primary mouse hepatocyte cultures, and levels of Par-4 are significantly increased in hepatocytes exposed to ethanol. Targeted knockdown of Par-4 by highly specific and effective small interference RNAs (siRNAs) inhibited mitochondrial dysfunction and caspase-3 activation induced by ethanol, and protected hepatocytes from apoptosis. These results identify Par-4 as a novel regulator of ethanol-induced hepatocyte apoptosis, and suggest that targeted inhibition of Par-4 by RNAi may prove to be an effective cytoprotective strategy against alcohol-induced hepatotoxicity.

1449

**The Effect of Mutated Lamin A/C Proteins on the Function of Emerin in Cells Isolated from AD-EDMD Patients**

L. J. Emerson,[1] M. Wehnert,[2] J. A. Ellis[1]; [1]The Randall Division of Cell and Molecular Biophysics, Kings College London, London, United Kingdom, [2]Institute of Human Genetics, Greifswald, Germany

Muscular Dystrophies are a family of progressive skeletal muscle wasting diseases of varying severities caused by defects in a number of different genes. Emery-Dreifuss Muscular Dystrophy (EDMD) also shows a severe cardiac phenotype which can cause sudden cardiac death. There are two forms of EDMD, an X-linked form which is caused by the absence or mutation in the nuclear envelope protein emerin, and an autosomal dominant (AD) form which is caused by mutations in the nuclear lamina proteins lamins A and C which are alternatively spliced from the same gene. Emerin is ubiquitously expressed but shows higher levels of expression in both skeletal and cardiac muscle. It is thought to have a role in maintenance of nuclear membrane structure, cell cycle mediated events and regulation of gene expression. Emerin and lamin A/C proteins have been shown to interact with each other as part of a macro protein complex and are co-localised in myoblasts. The aim of this research is to investigate the effects of EDMD-causing lamin mutations on the function of emerin in cell cycle mediated events using patient skin fibroblast cells lines. These cell lines from EDMD patients each contain a different lamin A/C mutation: R249Q and R377H in the central rod domain, and R401C and R453W in the emerin binding region. Our studies suggest that our AD-EDMD cell lines express less lamin A/C protein, which also exhibit a reduced strength of interaction to nuclear components. Similarly, emerin in these patient cell lines was more soluble. Two of our AD-EDMD cell lines exhibited altered rates of proliferation, suggestive of an extended cell cycle length.

1450

**Genomic Instability in Laminopathy-based Premature Aging**

Z. Zhou; Biochemistry, University of Hong Kong, Hong Kong, Hong Kong Special Administrative Region of China

Premature aging syndromes often result from mutations in nuclear proteins involved in the maintenance of genomic integrity. LAMIN A is a major component of the nuclear lamina and nuclear skeleton. Truncation in LAMIN A causes Hutchinson-Gilford Progerial Syndrome (HGPS), a severe form of early onset premature aging. Lack of functional ZMPSTE24, a metalloproteinase responsible for the maturation of Prelamin A, also results in progeroid phenotypes in mice and humans. To understand the molecular mechanism underlying HGPS, we investigated DNA damage and repair in mice lacking Zmpste24 and in cells from patients with HGPS. We found that *Zmpste24*-deficient mouse embryonic fibroblasts (MEFs) exhibit increased DNA damage and chromosome aberrations and are more sensitive to DNA-damaging agents. Bone marrow cells isolated from *Zmpste24*$^{-/-}$ mice display increased aneuploidy and the mice are more sensitive to DNA damaging agents. Recruitment of p53 binding protein 1 (53BP1) and Rad51 to sites of DNA lesions is impaired in *Zmpste24*$^{-/-}$ MEFs and in HGPS fibroblasts, resulting in delayed checkpoint response and defective DNA repair. Wild-type MEFs ectopically expressing unprocessible Prelamin A exhibit similar defects in checkpoint response and DNA repair. Our results indicate that unprocessed Prelamin A and truncated LAMIN A act dominant negatively to perturb the DNA-damage response and repair, resulting in genomic instability which might contribute to laminopathy-based premature aging.

1451

**Skeletal Muscle Fibrosis in Duchenne Muscular Dystrophy: Modulation by TGFβ and CTGF in *mdx* Mice**

V. Mezzano, D. Cabrera, E. Brandan; Cell & Mol. Biol., Catholic University of Chile, Santiago, Chile

Duchenne muscular dystrophy (DMD) is a lethal, muscle wasting disease caused by a mutation in the dystrophin gene. The absence of the dystrophin protein leads to degeneration of muscle fibers and eventually fibrotic scarring ensues. The *mdx* mouse model of DMD also lacks dystrophin and its diaphragm reproduces the degenerative changes of DMD. The aim of our work is to understand the fibrotic process which parallels muscle degeneration and, particularly, the involvement of two potent regulators of extracellular matrix (ECM) synthesis: TGFβ and Connective Tissue Growth Factor (CTGF), which has been described as another profibrotic cytokine. We have isolated fibroblasts from *mdx* and control mouse diaphragm, studied their relationship to these factors both regarding their expression and synthesis, and their regulation of ECM molecules. Here we report that CTGF induces fibronectin in fibroblasts of control mice in a concentration dependent manner. Regarding *mdx* fibroblasts we observed that they express and synthezise more CTGF than controls. We, therefore, studied ECM molecules present in these cell cultures and observed an increase in fibronectin and chondroitin sulfate proteoglycans in *mdx* fibroblasts compared to control, increase which is not observed in skin isolated fibroblasts. Interestingly, regarding the ability of proteoglycans to influence the activity/bioavailability of TGFβ, we observed increased binding of TGFβ to TGFβ Receptor III, or betaglycan, a heparan sulfate proteoglycan. These results suggest a constitutive activation of *mdx* fibroblasts obtained from dystrophic muscle in spite of their being isolated from their regenerative/fibrotic niche offering new insights into the understanding of these disorders. (Supported by MDA 3790, FONDAP-MIFAB, PG53/04 School of Medicine PUC, CONICYT AT-24050106)

1452

**Development of Dual-reporter Transgenic Mice for Readthrough Assay**

M. Shiozuka, K. Shimada, R. Matsuda; Dept. of Life Sci., The Univ. of Tokyo at Komaba, Tokyo, Japan

Negamycin is a naturally-occuring dipeptide antibiotic that causes the RNA translation mechanism in the ribosomes to ignore frequently such a premature termination codon (PTC), i.e. to readthrough it. We previously reported that negamycin rescues dystrophin expression in mdx skeletal and cardiac muscles with less side effects than gentamicin (J. Biochem., 2003). We suggest that negamycin is a therapeutic candidate for not only Duchenne muscular dystrophy but also other genetic disorders caused by nonsense mutations. In order to investigate more potent readthrough stimulator without toxicity and facilitate studies, we have developed a new in vivo dual- reporter assay system for measuring readthrough efficiency. Dual-reporter gene is composed with beta-galactosidase gene and luciferase gene connected with PTC region (27mer which contains the sequence around the PTC of exon 23 of mdx mouse dystrophin) driven by cytomegalovirus/beta-actin hybrid promoter. This dual-reporter allows a simultaneous sensitivity and quantitative accuracy. We have established three transgenic (Tg) mouse strains containing different PTCs (Ochre [TAA], Amber [TAG], Opal [TGA]), that express beta-Galactosidase in skeletal muscle, cardiac muscle and brain. Using these Tg mice, we succeeded in the evaluation of readthrough activity in each tissue in vivo and in real-time in vitro for the first time. Furthermore, we tested the efficacy of cutaneous antibitic ointment in Tg mice. The results showed that treatment regimens that included topical gentamicin provided better rates of readthrough than treatment regimens in which only base preparations were used. We will evaluate this therapeutic potential for variety of other genetic disorders. This work was supported by a Research Grant for Nervous & Mental Disorders and by a grant for Research in Brain Science from the Ministry of Health, Labor and Welfare, Japan.

1453

**Acidic Sphingomyelinase has a Dual Role in Modulating Apoptosis Induced by Reactive Nitrogen Species in Airway Epithelial Cells**

S. Castillo, M. Levy, R. Lanir, C. Wang, J. Vihas, T. Goldkorn; Internal Medicine, University of California at Davis, Davis, CA

Reactive nitrogen species (RNS) are highly reactive molecules that have been implicated in the pathophysiology of inflammatory lung diseases, such as acute respiratory distress syndrome, asthma, and chronic obstructive pulmonary disease. Recently studies have suggested a role for acidic sphingomyelinase (aSMase) and ceramide in lung diseases such as pulmonary edema and emphysema. Previously, we reported that both peroxynitrite (ONOO-) and nitric oxide (NO) generated ceramide in lung epithelial cells, but that only ONOO- induced apoptosis. We found that ONOO- generated ceramide through the activation of acidic sphingomyelinase (aSMase) whereas NO generated ceramide through the activation of ceramide synthase. The objective of the current study was to determine how different RNS modulate apoptosis in lung epithelial cells by examining the regulation of components of the ceramide generating machinery such as aSMase. siRNA impairment of aSMase reduced basal and inducible enzyme activity by 60% and 50% respectively. Furthermore, knockdown of aSMase reduced ONOO- induced apoptosis by approximately 40%. In addition, inhibition of a downstream caspase, caspase-3, also resulted in an inhibition of ONOO- induced apoptosis by ~40%, suggesting that ceramide generation by ONOO- exposure precedes caspase-3 activation. As mentioned previously, despite the fact that ceramide was generated by NO treatment, no apoptosis was observed. We found that upon NO exposure, an increase in the protein-protein interaction between aSMase and caspase-3 occurred, with aSMase sequestering caspase-3 and preventing its cleavage by upstream caspases and thereby blocking an apoptotic response. Knockdown of aSMase resulted in caspase-3 cleavage and the induction of apoptosis after NO treatment. These studies demonstrate a novel way RNS regulate apoptosis in lung epithelial cells by modulating the function of acidic sphingomyelinase to be either a pro-apoptotic mediator or an anti-apoptotic molecule.

1454

**Development and Characterization of the Cystic Fibrosis Airway Epithelial Cell Lines Complemented with wild-type and F508del CFTR cDNA**

R. Maurisse,[1] J. W. Cheung,[1] B. Illek,[2] H. Fischer,[2] T. Shuto,[3] D. C. Gruenert[1]; [1]Research Institute, California Pacific Medical Center, San Francisco, CA, [2]Children's Hospital Oakland Research Institute, Oakland, CA, [3]Faculty of Medical and Pharmaceutical Sciences, Department of Molecular Medicine, Kumamoto, Japan

The development of immortalized airway epithelial cells is critical in elucidation the biochemical and molecular mechanisms underlying cystic fibrosis (CF) pathology and in the development of CF therapies. Different cell systems have been employed in genetic complementation studies and have been used to characterize CF transmembrane conductance regulator (CFTR) -related metabolic pathways. An important feature of CF

cells complemented with wild-type (WT)-CFTR is the reversal of the cAMP-dependent Cl ion transport defect in these cells. Such complementation studies have led to the understanding of the involvement of CFTR function in specific biochemical pathways. Numerous CF cells have been complemented with CFTR cDNA with ~4.7 kB open reading frame (ORF) CFTR cDNA or the entire ~6.2 kB CFTR cDNA comprised of both the ORF and the 3' and 5' untranslated regions (UTR). The studies described here evaluate CF airway epithelial cells that have been complemented with the 4.7 kB, 6.2 kB WT-CFTR, and the 4.7 kB F508del-CFTR under the regulation of a cytomeglovirus (CMV) promoter and cloned into an Epstein Barr Virus (EBV) -derived episomal expression vector. Cell lines that are either homozygous for the F508del mutation or are compound heterozygotes (F508del/Q2X) have been developed and evaluated for their CFTR expression levels and their ion transport characteristics. CF cells complemented with wt 6.2 kB cDNA which exhibit comparable level of WT expression in wt cells line (16HBE14o-) have similar Cl transport activity as the wt cell line. Further studies are underway to more fully correlate the degree of CFTR expression with the ion transport characteristics of the complemented cells.

1455

**Redox Potentials in the Cytosol, ER, Mitochondria and Membrane Surface of CF and CFTR-Corrected Airway Epithelia Measured with roGFP**

C. Schwarzer,[1] H. Fischer,[2] B. Illek,[2] C. Dooley,[3] S. Remington,[4] R. Tsien,[3] T. Machen[1]; [1]Molecular and Cell Biology, Univ California, Berkeley, CA, [2]CHORI, Oakland, CA, [3]UCSD, La Jolla, CA, [4]Univ. Oregon, Eugene, OR

There are many potential contributors to altered redox balance cystic fibrosis (CF) airways: (i) Loss of CFTR and its ability to transport glutathione may lead to oxidation of the airway surface liquid (ASL). (ii) Upregulated Na transport in CF may cause hypoxia and increase mitochondrial production of reactive oxygen species. (iii) Altered CFTR processing in the endoplasmic reticulum (ER) may cause ER stress. We studied the role of CFTR in controlling redox balance in airway epithelial cells by targeting ratiometric, redox-sensitive GFP (roGFP1) to the cytosol, ER, mitochondria and apical surface in the CF nasal cell line JME and also in JME cells in which CFTR had been expressed using an adenovirus. Quantitative measurements were performed using ratio imaging microscopy including calibrations of fluorescence signals in terms of redox potentials measured with a redox-sensitive electrode. Steady state redox potentials were > -50 mV in the ER and at the cell surface, -220 mV in the mitochondria and -250 to -280 mV in the cytosol. These compartments exhibited redox regulation in that their redox potentials recovered, though with quite different time courses depending on the organelle, following brief pulses with either oxidants (H2O2) or reductants (dithiothreitol). As measured using Ussing chambers and electrophysiology, CFTR expression elicited increases in apparent forskolin-stimulated Cl secretion and small decreases in Na absorption, but neither steady state redox potentials nor redox regulation were altered. These data show for JME cells that the ER and membrane surface are oxidized compared to the cytosol and mitochondria, and rates of redox regulation in these compartments are all different from each other. However, CFTR seems not to play a direct role in their redox regulatory properties. Support: CFF, NIH, HHMI

1456

**Integrin Alpha V Beta 5 Regulates Lung Vascular Permeability and Acute Lung Injury by Organizing Signaling Complexes at Endothelial Adherens Junctions**

G. Su,[1,2,3] J. A. Frank,[4,3] M. Hodnett,[5,6] N. Wu,[1] M. Godzich,[5,6] M. A. Matthay,[2,3,5] D. Sheppard,[1,2,3] J. Pittet[5,6]; [1]Lung Biology Center, UCSF, San Francisco, CA, [2]Cardiovascular Research Institute, UCSF, San Francisco, CA, [3]Pulmonary and Critical Care Medicine, UCSF, San Francisco, CA, [4]San Francisco VA Medical Center, San Francisco, CA, [5]Departments of Anesthesia and Surgery, UCSF, San Francisco, CA, [6]Laboratory of Surgical Research, UCSF, San Francisco, CA

Acute lung injury (ALI) is a common, currently untreatable disorder with a high mortality rate. We now show that in two clinically relevant models of lung injury (ischemia- reperfusion and ventilator-induced lung injury), blockade or genetic ablation of integrin alpha v beta 5 dramatically reduces the increase in pulmonary vascular permeability that is the central physiologic feature of ALI. Although integrins are best characterized for their role in attaching cells to the underlying extracellular matrix, in pulmonary endothelial cells, alpha v beta 5 is principally localized to cell-cell junctions, where it is part of a large protein complex that also contains the adherens junction protein, VE-cadherin, the small GTPase RhoA, and the RhoA upstream activator p115rhoGEF. *In vitro* increases in pulmonary vascular permeability induced by three distinct signaling pathways activated by VEGFA, TGFβ and thrombin are each prevented by inhibition of both RhoA and alpha v beta 5. Furthermore, both RhoA and alpha v beta 5 appear to regulate induction of actin stress fiber formation and phosphorylation of VE-cadherin induced by each agonist. These results suggest a novel mechanism by which alpha v beta 5 coordinates increases in pulmonary vascular permeability in response to multiple agonists by regulating endothelial junction protein activation and cell shape changes. We hypothesize that alpha v beta 5 may control local RhoA activation at pulmonary endothelial adherens junctions by coordinating accessibility to activators of RhoA like p115RhoGEF and therefore, might be an attractive target for pharmacologic intervention in ALI.

1457

**QRT-PCR Gene Expression Analysis of Nitric Oxide Synthase in COPD Patients and Normal Individuals Using Formalin-Fixed Paraffin-Embedded Lung Tissues**

N. Novoradovskaya,[1] N. T. Eissa,[2] A. Novoradovsky,[1] J. Braman,[1] F. Kheradmand[2]; [1]R&D, Stratagene, La Jolla, CA, [2]Baylor College of Medicine, Houston, TX

Nitric oxide synthases (NOS) are a family of enzymes that control the production of nitric oxide by converting L-arginine to L-citrulline. It is known that nitric oxide concentrations are elevated in patients with severe asthma, cystic fibrosis, or in patients with chronic obstructive pulmonary disease (COPD). Our prior work (Novoradovsky et al., 1999) demonstrated a modulatory role for endothelial NOS isozyme in destructive lung disease associated with alpha-1antitrypsin deficiency. In this study we compared the gene expression level of neuronal, inducible and endothelial nitric oxide synthases (nNOS, iNOS and eNOS) in the lung tissues of COPD patients and normal individuals. Total RNA was isolated by Absolutely RNA FFPE kit (Stratagene) from formalin-fixed paraffin-embedded (FFPE) lung tissue obtained from four normal and four COPD patients. Gene expression level of nNOS, iNOS and eNOS was evaluated by QRT-PCR using TagMan Probes (ABI) and the Mx3000P Real-time PCR System (Stratagene). β2-Microglobulin (B2M) was used as the normalizing gene. Standard curves were generated using QPCR Universal Human Reference RNA. Relative mRNA quantification was performed using the software application for the "Comparative Quantification"

method on the Mx3000P.The quantity and quality of RNA isolated from FFPE lung tissues by Absolutely RNA FFPE kit were sufficient for QRT-PCR analysis. No difference in gene expression levels of nNOS or iNOS between the two groups were observed. Expression of eNOS was decreased by 2-3 fold in COPD patients compared to healthy controls. Decline in eNOS expression in patients with COPD indicates possible involvement of this izozyme in pulmonary disease. Alternatively, reduced expression of eNOS might result from destruction of endothelial and airway wall tissues in the lung samples from COPD patients.

1458

**Ligation of PAR-1 Enhances αvβ6 Integrin Dependent TGFβ Activation and Promotes Acute Lung Injury**

G. Jenkins,[1] X. Su,[2] G. Su,[3] E. Camerer,[2] G. J. Laurent,[1] G. E. Davis,[4] R. C. Chambers,[1] M. A. Matthay,[2] D. Sheppard[3]; [1]Centre for Respiratory Research, University College London, London, United Kingdom, [2]Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA, [3]Lung Biology Center, University of California, San Francisco, San Francisco, CA, [4]Department of Pathology and Laboratory Medicine, Texas A&M University System Health Science Center, College Station, TX

Activation of latent TGFβ by the αvβ6 integrin is a critical step in the development of acute lung injury. The αvβ6 integrin must be activated at the onset of acute lung injury, but the mechanisms of activation have not been previously identified. The serine protease, thrombin, is generated early during acute lung injury and is thought to contribute to increased lung permeability via its ability to activate PAR-1. We used two readouts of TGFβ activity, phosphorylation of SMAD2 and a co-culture bioassay, to examine the effects of thrombin and the PAR-1 activating peptide SFLLRN on TGFβ activation by lung epithelial cells. Both agonists increased TGFβ activity in both assay systems and these effects were inhibited by an αvβ6 blocking antibody, demonstrating that ligation of PAR-1 induces αvβ6-dependent TGFβ activation. SFLLRN had no effect on TGFβ activation in PAR-1-/- fibroblasts engineered to express the αvβ6 integrin. Infection of αvβ6-expressing fibroblasts with adenoviral dominant negative RhoA inhibited the response to SFLLRN, whereas constitutively active RhoA enhanced αvβ6 integrin mediated TGFβ activation, even in PAR-1-/- cells. αvβ6 integrin mediated TGFβ activity was also inhibited by the Rho kinase inhibitor Y-27632. To determine the relevance of this pathway in vivo we ventilated mice at high tidal volumes following intratracheal instillation of the PAR-1 agonist TFLLRN or the control peptide FTLRRN. Under the conditions studied, lung permeability was doubled by TFLLRN, an effect that was inhibited by an αvβ6 integrin blocking antibody. The increases in lung permeability and nuclear phosphoSMAD2 staining induced by intratracheal instillation of bleomycin were also significantly reduced in PAR-1-/- mice. These data demonstrate that PAR-1 stimulation leads to αvβ6 mediated TGFβ activation via RhoA and that this pathway is important in the development of acute lung injury.

1459

***"Non-Coding"* Small RNA Bioaptamers as Codes for Epigenetic Phenotype Alterations in Proteinaceous Transfer of Delayed-Type Hypersensitivity and Functions in Wound Healing: RNA-Operated Conformation Phase Pathway-Locked Loops [CPLL] in Epigenomes**

J. H. Wissler,[1] J. E. Wissler,[1] E. Logemann[2]; [1]ARCONS Institute for Applied Research & Didactics, Postfach 1327, Bad Nauheim, Germany, [2]University of Freiburg, Freiburg, Germany

OBJECTIVE: Functions of entitled structure-defined *endogenous* RNA as signals in epigenetic [non-Mendelian] phenotype alterations [EPA] by proteinaceous transfer factors of delayed-type hypersensitivity [TF-DTH] and wound healing. METHODS: Isolation of such RNA from extracellular wound and supernatant fluids of quiescent, lectin- or antigen-activated normal and sensitized cells cultured in serum-free synthetic media. Purification guided by bioassays of cellular differentiation and tissue morphogenesis/ wound healing in terms of non-mitogenic angio-morphogenesis, negative regulation [inhibition] of granulopoiesis and leftward shift reactions [Wissler et al., Protides Biol. Fluids 34:517-536,1986; Materialwiss.Werkstofftech.32:984-1008,2001; Arch.Surg.124:693-698,1989; Ann.NY.Acad.Sci.961:292-297,2002; 991:333-338,2003; 1022:163-184,2004; FASEB J.19:A621,2005; FEBS J.272-s1:N1-06-4P,2005]. RESULTS: From extracellular TF-DTH preparations, bioactive *endogenous* complexes of nucleic acid bioaptamers and protein conformers could be isolated. Some sequence-defined RNA bioaptamers are redox- and metalloregulated, edited and modified "*non-coding*" small RNA [<200 bases] as regulators and messengers of protein structure [folding] at cellular translation and transcription. They address conserved homologous metalloregulated RNA-binding domains in epigenetic regulator proteins [e.g. of angio-morphogens like angiotropin], termed K/R3H-[K/RxxxH], i.e. -t/s/xK/R/q/nxxxH/y/n/q/e/dx$_{7\text{-}9}$h/xx$_{7\text{-}9}$h/xx$_{5\text{-}20}$K/R/q/n/e/h- with accessory basic [R/K]$_n$, R/K-zipper, SR/K/RS and/or HxxxH/y/n/q segments. Sequences and positions may suggest that only some EPA are transmissible or prone to "*bioaptamer disease*" and as upon some protein maturation, copper-binding ATCUN motifs may be formed. CONCLUSIONS: Since not directly retranslatable to heritable genome codes by sequence edition, base modification, redox- and metalloregulation, i.e. code extensions/alterations, entitled *"non-coding"* RNA bioaptamers as products of overwhelming portions [~98%] of transcriptional output apparently represent part of cryptic codes for EPA in DTH transfer and investigated wound healing functions. CPLL orchestrated by *"non-coding"* RNA, redox and metal ions are suggested mechanisms for reprogramming cell fate in EPA of DTH and wound healing reactions.

1460

**Effect of Bioelectromagnetic Fields on Wound Healing in the Sea Urchin, *Lytechinus variegatus***

K. A. Kedrowicz,[1] J. Mckeague,[1] A. Sturycz,[1] D. F. Johnson,[2] L. Winslow[1]; [1]Biology, Northwestern College, St. Paul, MN, [2]Psychology, Northwestern College, St. Paul, MN

All humans emit very low frequency electromagnetic fields known in the literature as bioelectromagnetic fields (BEMFs). Using the sea urchin model, we investigated the effect of BEMFs on wound healing in the sea urchin *Lytechinus variegatus*. Animals were wounded producing a longitudinal 2cm x 0.5 cm incision along one seam of the test. BEMFs were applied by a trained practitioner for 10 minutes each day over five days. Controls maintained in a separate aquarium, were wounded similarly, but did not receive BEMF treatments. Wounds on control and experimental animals were measured daily for longitudinal wound closure. Our data showed that wounds closed faster and more completely in BEMF treated groups than in controls. Measurements of wounds in the BEMF treated group were 1.7cm, 0.75 cm, 0.5 cm on days 2, 4 and 6 respectively, compared with 1.9cm, 1.5cm, 1.3 cm on the same days in the control. This corresponds to a nearly 80% rate of wound healing in the experimental compared to 35% in controls. Student's t Test analysis applied to the data showed statistical significance of $p<.005$. We also observed that animals treated with BEMFs showed remarkably enhanced vitality and longevity over corresponding controls. Immune cell recruitment into the wounds was examined in BEMF treated and untreated animals over five days. BEMF treated animals possess enhanced migration of red

spherule and colorless spherule immune coelomocytes into the wounds by day two after wounding. Similar recruitment of immune cells occurred on day four in controls. We also noticed an increased number of test spicules in BEMF treated animals indicating a stimulation of calcium carbonate secreted by immune effector cells. Taken together, our data indicate that BEMFs significantly enhance immune cell activation and recruitment accelerating wound healing pathways.

1461

**Silver Sulfadiazine and β-sistoserol in Burn Wound Healing: A Comparative Study**

A. R. Jurjus,[1] A. A. Gerges,[2] M. Abou-Jaoudeh,[2] I. Abdallah,[1] R. Tohmé,[1] R. Jurjus,[1] S. Hayek,[1] B. Atyieh[1]; [1]Human Morphology, American University of Beirut, Beirut, Lebanon, [2]Faculty of Pharmacy, Saint Joseph University, Beirut, Lebanon

Healing of cutaneous wounds is a complex process whereby an array of topical agents is frequently used. Antiminobials, have been shown to be cytotoxic to fibroblasts and keratinocytes and to retard wound healing. While others have been considered as promoting wound healing especially when they modulate cytokines. This study was designed to compare the effect of silver sulfadiazine and β-sistosterol creams on burn wound healing in rabbits with thermal burn injury. The rabbit model of Knobl et al., 1999 was adopted . Nine adult male rabbits, average weight 2500g, were divided into 3 groups (G). They were depilated to obtain an absolutely smooth and hairless dorsal skin and anesthetized by ketamine and xylasine. The thermal burn injury was performed on 4 spots of the dorsal skin using a modified soldering iron at a temperature of 80$^{o}$C for a duration of 22 seconds resulting in deep partial thickness burn of 3.8 cm$^{2}$. Lesions biopsies with surrounding healthy skin were excised daily and either fixed in 5% formaldehyde for histological studies or frozen for cytokine studies (b-FGF and TGF-β) by ELISA. G1 was treated with saline, G2 with MEBO and G3 with silver sulfadiazine. The dressings were changed daily. Transepidermal water loss (TEWL) was measured and observations recorded daily. Results showed that the least TEWL was in the MEBO group and the highest was in the silver sulfadiazine group. Histological findings depicted a relatively faster healing (Δ 3 days) in G2 compared to G1 and G3 in addition to a better healing quality (less depression and less surface alterations) compared to the other two groups. Some cytokines were modulated, in particular, bFGF increased in G2. Further quantification of cytokines IL-1, TGF-β are being carried out. (Grant from The MPP, AUB)

1462

**Cholesterol Stabilizes Rab9 on Late Endosomes of Niemann-Pick type C Fibroblasts**

I. G. Ganley, S. Pfeffer; Biochemistry, Stanford University, Stanford, CA

Niemann-Pick type C (NPC) is an intracellular trafficking disorder resulting in the accumulation of cholesterol and glycosphingolipids in late endosomes and lysosomes. It has been reported that overexpression of the small GTPase Rab9 relieves this accumulation (1,2). This suggests that normal Rab9 function may be compromised in NPC cells and/or its overexpression drives a bypass transport pathway out of late endosomes, thus providing an exit route for excess cholesterol. To help understand how Rab9 overexpression rescues cholesterol accumulation, we have investigated endogenous Rab9 in NPC cells. Rab9 levels were elevated 1.8 fold in NPC cells relative to wild type and the half life increased 1.6 fold, suggesting accumulation of Rab9 is due to slower degradation. Increased half life is likely due to stabilization of Rab9 on endosomes as shown by a reduction in GDI-mediated Rab9 extraction from NPC membranes compared to wild type. Why should increased cholesterol stabilize membrane-bound Rab9? It is possible that the Rab9-associated prenyl groups interact with higher affinity with cholesterol-rich bilayers. Alternatively, the Rab may be stabilized by an effector that is itself stabilized on endosomes by increased cholesterol. Salt washing of endosomes, to remove peripheral proteins, or RNAi of TIP47 (a Rab9 effector whose level also increased on cholesterol-laden endosomes) failed to relieve the block in Rab9 extraction. Thus, peripherally associated proteins do not seem sufficient to explain Rab9 stabilization. Evidence for a direct role of cholesterol in stabilizing Rab9 was obtained using liposomes loaded with prenylated Rab9: increasing the cholesterol content of these liposomes resulted in decreased Rab extractability. We conclude that cholesterol contributes directly to the stabilization of Rab9 on membranes of NPC cells. (1) Choudhury et al. (2002) J Clin Invest. 109:1541-50. (2) Walter et al. (2003) J Lipid Res. 44:243-53.

1463

**A Novel Antiobesity Compound BLX-1028 Inhibits iNOS and Adipocyte Differentiation**

M. Anumula, S. Roy, A. Nag, P. Neogi, B. Nag, D. Dey; Bexel Pharmaceuticals Inc, Union City, CA

Obesity is a condition of excessive fat in the body and the adipocytes are the major sites for the storage of fat. Controlling either increase in the adipocyte cell size or differentiation might be a good strategy to control the weight gain. It is known that iNOS (Inducible Nitric-oxide Synthase) expression is highly elevated in muscle and fat of obesity animal models. We have made several amino acid conjugated small molecules and screened them in both anti-adipogenic and anti-inflammatory assays. We identified BLX-1028 as a unique molecule with both antiobesity and anti-inflammatory properties. Adipogenesis was induced by incubating the cells with rosiglitazone (1uM), a known PPARγ agonist, and co-treated with 0.1, 1 and 10uM concentrations of BLX-1028. Hormone mediated adipogenesis was induced by a mixture of IBMX (3-Isobutyl-1-methylxanthine), dexamethasone and insulin, and treated with different concentrations of BLX-1028. The cells were stained with Oil Red O and visualized under Olympus microscope. Oil Red O was extracted to quantify the amount of triglyceride accumulation. In both experiments BLX-1028 showed dose dependent inhibition of TG accumulation. At 10 uM it showed 90% ($P<0.05$) inhibition of TG. It also inhibited LPS and cytokine induced iNOS expression in different cells. At 10 and 30 uM it showed 70% and 90% inhibition of nitrate in 3T3-L1 adipocytes compared to vehicle, as measured by Griess reagent. In hPBMC cells, compared to vehicle, BLX-1028 lowered TNF-α level by 34, 70 and 84 % at 3, 10 and 30uM concentrations respectively. Based on the above results we conclude that BLX-1028 is a novel molecule with a unique mechanism of targeting adipocytes, which makes it completely different from all the marketed antiobesity drugs. Currently BLX-1028 is being tested in different animal models for obesity.

1464

**Inhibitory Effect of Magnolia Officinalis on Aortic Oxidative Stress and Apoptosis in Hyperlipidemic Rabbits**

W. Chang, Y. Hsu, C. Wu, Y. Yu, P. Yin, S. Chiang; China Medical University, Taichung, Taiwan Republic of China

Twenty hyperlipidemic rabbits were served one of the following: a high-fat and cholesterol diet (cholesterol group, 10% corn oil and 0.5% cholesterol), a high fat and cholesterol diet supplemented with Magnolia officinalis extract (300 mg/kg) or lovastatin (6 mg/kg). The plasma lipids, oxidative stress (measured by free radical, MDA and oxidative DNA damage) and lipid lesions significantly decreased in the Magnolia officinalis

and lovastatin groups when compared with the cholesterol group. Moreover, the expressions of Fas-Ligand, caspase 8 and caspase 9 in the aortic arches were also markedly lowered after Magnolia officinalis and lovastatin supplements. Therefore, the results indicate that anti-atherogenic effect of Magnolia officinalis is involved with a suppression of oxidative stress and with the down-regulation of apoptosis related gene expression in hyperlipidemic rabbits.

## Reprogramming Cell Fate (1465-1467)

1465

### Reprogramming Cell Fate by Nuclear Transfer

J. B. Gurdon; Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, United Kingdom

The differentiated state of adult somatic cells is very stable. Only very occasionally do cells that have been committed to one pathway of cell fate change directions and enter a different specialization pathway. However, if the nuclei of such determined or differentiated cells are transplanted to eggs or oocytes of amphibia or mammals, a dramatic reversal can take place. In these cases, somatic cell nuclei are rejuvenated to an embryonic pattern of gene expression within only a few hours. As cells differentiate the ease with which they can be reprogrammed by egg cytoplasm declines. In some of these cases somatic cell nuclei retain a memory of their previous pattern of gene expression for numerous cell divisions. The changes that are induced by egg or oocyte cytoplasm fall into three classes. (i) The removal of marks that characterize the differentiated state of a nucleus. These include the demethylation of genomic DNA in the promoter region of genes that undergo reprogramming. Also, modified proteins associated with the inactive state of genes are removed. (ii) The provision by host cells of those transcription factors required to read genes that undergo activation. (iii) The decondensation of chromatin to provide access of transcription factors to the regulatory region of activated genes. The large size and abundance of Xenopus eggs and oocytes facilitates identification of the molecules and mechanisms involved in reprogramming somatic cell nuclei. The long-term aim of this direction of work is to pre-treat somatic cells with reprogramming agents in order to increase the efficiency with which nuclei can be reprogrammed to an embryonic state. The derivation of embryonic stem cells from nuclear transplant embryos could lead to a cell replacement therapy that avoids the need for immunosuppression.

1466

### Regulating Cell Fate by Fusion

H. M. Blau; Baxter Laboratory in Genetic Pharmacology, Stanford University, Stanford, CA

Cell fusion has long been recognized as an experimental means for altering the gene expression profile from that of one cell type to that of another. The plasticity of cell fate observed in such heterokaryons in tissue culture is well known to result largely from the balance of regulators within the cell at any given time. Recently, in response to injury, hematopoietic stem cell (HSC) derivatives have been shown to be capable of fusing with non-hematopoietic tissues in vivo in mice and humans. To contribute function after fusion, reprogramming of the donor nucleus is critical, a process that has been demonstrated to occur to some extent, but which remains inefficient. We are taking several approaches to investigate the molecules and regulatory networks underlying the reprogramming of diverse mammalian somatic cell nuclei for distinct differentiated fates after fusion. (1) Perturbing chromatin remodeling of enhancer/promoter elements in tissue-specific genes by manipulating histone deacetylase activity; (2) Analyzing changes in the methylation state of genomic sequences that influence the degree and rate of nuclear reprogramming; (3) Determining to what extent the stage of differentiation and associated repression of alternative gene expression programs influences the efficiency of nuclear reprogramming. The ultimate goal of this research is twofold. First, the in vivo potential of HSC derivatives to contribute to adult tissues to which they fuse spontaneously may be enhanced which would advance the field of regenerative medicine. Second, the in vitro reprogramming of the nuclei of somatic cells derived from diverse sources to function as differentiated tissue-specific cells could yield determined stem or progenitor cells for transplantation and use as replacement therapy for defective host cells. Ideally, cell fusion would constitute a means of post-natal cloning, by-passing the need for eggs or for directing the differentiation of ES cells.

1467

### Cell Fusion and Mitotic Reduction Divisions in Murine Epithelial Cells

M. Grompe,[1] H. Willenbring,[1] M. Al-Dhalimy,[1] M. Finegold[2]; [1]Department of Medical & Molecular Genetics, Oregon Health and Science University, Portland, OR, [2]Baylor College of Medicine, Houston, TX

Work from several laboratories has shown that hematopoietic stem cells (HSC) can give rise to epithelial cell types in the liver and kidney. Bone marrow derived hepatocytes (BMH) can successfully revert hepatic dysfunction in an animal model of a genetic liver disease, hereditary tyrosinemia type 1 (HT1). Similarly, bone marrow derived cells can reconstitute the tubular epithelium of the kidney in this model. These observations have raised the question whether bone marrow transplantation may have broad utility for the treatment of hepatocellular and renal diseases. However, recent experiments showed that the majority of bone marrow derived epithelial cells (BME) arise by cell fusion, not differentiation of stem cells. BMH express hepatocytic, but not hematopoietic markers and are fully functional in rescuing liver function in vivo. Therefore, the production of HSC derived epithelial cells involves at least partial reprogramming of the nucleus of the hematopoietic fusion partner to the hepatocyte fate. Transplantation of purified subpopulations of hematopoietic cells demonstrated that mature myelomonocytic and not stem cells were the fusion partner. Importantly, cells derived from fusion between hematopoietic donor cells and recipient epithelial cells were able to divide despite being tetraploid. We utilized serial transplantation of BMH to analyze the effects of cell division on the karyotype of synkaryons. Female bone marrow donors were transplanted into male HT1 recipients, followed by serial transplantation of BMH into female secondary recipients. Cytogenetic analysis revealed abundant numbers of male diploid BMH in the female secondary recipients. This observation strongly suggests that the tetraploid synkaryons underwent mitotic reduction divisions. Mitotic reduction divisions of epithelial cells have previously been described in insects but not in mammals. The frequency and significance of this phenomenon are currently unknown.

## Community Building to Promote Careers in Biomedical Science (1468)

1468

### Community Building to Promote Careers in Biomedical Science

C. H. Evans, M. I. Klopf, A. L. French; Department of Human Science, Georgetown University, Washington, DC

We have developed a rural underserved community partner education model to stimulate interest of high school students in biomedical science by working with students, their school, and community mentors. The goals are to 1) increase engagement of individuals from rural areas in the

intellectual and work life of biomedical scientists and health care professionals and 2) test the usefulness of a technologically-driven education model to higher education institutions in drawing rural communities into the intellectually and culturally rich life of institutes of higher learning. 70 high school junior and senior students and adults (52 youth and 18 adults) from the Oglala Lakota Sioux American Indian Pine Ridge Reservation in SD (20 participants), the African-American and Cajun Assumption Parish, LA community (36 participants) and Mexican-American migrant farm worker communities in Florida and Texas (14 participants) traveled to Georgetown University during 2003-5 for 3-week summer science institutes. The on-campus residential experiences featured educational skill evaluation and science classroom and hands on laboratory instruction by Georgetown University faculty, students, and invited experts, as well as career exploration activities. Continuing biomedical science education throughout the year is provided through a biological virtual laboratory that offers students innovative exercises to build qualitative and quantitative science critical thinking and analysis, and communications skills. Validating the effectiveness of this biomedical science education model for increasing individuals in the biomedical and health care related pipeline is being pursued by measuring the success of student participants in entering college and evaluating the biological virtual laboratory as a useful tool for distance interaction, learning and quantitative assessment. Supported by a NIH NCRR Science Education Partnership Award.

## Host-Pathogen Interactions (1469-1471)

1469

### The Biochemistry of HIV Budding

W. I. Sundquist; Department of Biochemistry, University of Utah, Salt Lake City, UT

The HIV Gag protein coordinates viral trafficking, membrane binding, assembly, cofactor packaging, budding, and maturation. Late in the infectious cycle, Gag assembles on plasma and endosomal membranes to form enveloped particles that bud through the membrane. We, and others, have previously shown that the cellular protein, TSG101, binds a conserved PTAP motif within Gag and facilitates virus budding. TSG101 normally functions as part of the multi-protein complex, ESCRT-I, which helps sort ubiquitylated protein cargos into vesicles that bud into late endosomal multivesicular bodies (MVB). These, and other observations support the idea that HIV and other enveloped RNA viruses usurp the machinery of MVB vesicle formation to bud from cells. I will describe our ongoing studies aimed at identifying and characterizing the cellular machinery required for HIV-1 budding, with particular emphasis on the ESCRT-I and VPS4/LIP5 complexes. VPS4/LIP5 is a large ATPase that functions late in MVB vesicle formation and virus budding by binding and releasing the assembled ESCRT machinery. Our structural studies reveal that VPS4 proteins are Type I AAA ATPases with five distinct regions: an N-terminal MIT domain that binds ESCRT-III substrates, a large alpha/beta AAA ATPase domain with a canonical Rossmann fold, a smaller helical AAA ATPase domain, a three-stranded antiparallel beta domain that binds LIP5, and a C-terminal helix of unknown function. Our current biochemical studies are aimed at understanding how VPS4/LIP5 releases/remodels the ESCRT-III machinery during MVB vesicle formation and virus budding.

1470

### Host-pathogen Interactions : The *Listeria* Paradigm

P. Cossart; Institut Pasteur, Paris, France

Bacterial pathogens have evolved a variety of strategies to maximally exploit host cell components and their specific properties, such as the cytoskeleton plasticity, during infection. *Listeria monocytogenes* has emerged as one of the best models to address these issues. During infection, this food pathogen disseminates from the intestinal lumen, to the liver and spleen up to the brain and the placenta after crossing three barriers, the intestinal-, the blood-brain- and the placental barrier resulting in gastroenteritis, meningitis, and materno-fetal infections. In infected tissues, *Listeria* is intracellular owing to its striking property to trigger its own internalization into cells which are normally non phagocytic and in which it replicates, somehow protected from host defenses. At the cell level, entry results in the formation of a vacuole in which the bacterium resides transiently before escaping into the cytosol where it moves using an actin-based motility process. Bacteria then spread directly from cell to cell resulting in their dissemination in infected tissues. Over the last decades, classical genetics, post-genomics and cell biology approaches together with *in vivo* studies using animal models including transgenic mice have led to the identification of listerial factors critical for specific steps of the infectious process, of their cellular ligands and of the signaling cascades resulting from their interactions, revealing how sophisticated are the strategies used by a single organism to exploit host components to its own profit and how tight is are the regulations that lead to a succesful infection. The study of the various underlying mechanisms has significantly contributed to decipher unsolved questions in cell biology such as actin-based motility. Recent results from our laboratory highlight that *Listeria* unexpectedly hijacks the clathrin dependent endocytosis machinery for entry into cells. Whether these results are of general relevance is under investigation.

1471

### Something Old, Something New, Something Borrowed, Something GREEN  The Cell Biology of Apicomplexan Parasites

D. S. Roos; Department of Biology & Genomics Institute, University of Pennsylvania, Philadelphia, PA

The phylum apicomplexa includes *Plasmodium* (which causes malaria), *Toxoplasma* (a leading source of congenital neurological birth defects, and a prominent opportunistic pathogen afflicting immunodeficient patients), and thousands of other protozoan species. Beyond their clinical importance, these intracellular parasites provide fantastic biological probes, manifesting much of the complexity that characterizes eukaryotes, but in a stripped-down package amenable to experimental manipulation both *in vitro* and *in vivo*. Conserved features provide insight into the evolutionary origins and dynamic functions of eukaryotic organelles, while parasite-specific features highlight organellar diversity and suggest strategies for blocking the progress of parasitic disease. Studies on the parasite cytoskeleton -- required for motility, host cell invasion, and replication -- reveals a paucity of filamentous actin, a diverse array of tubulin-based structures, a critical set of intermediate filaments, and a remarkable method of mitotic replication, in which highly polarized daughter cells are assembled within the mother according to a rigorous schedule of organellar duplication and segregation. Subcellular organelles include a specialized secretory apparatus that is required for host cell attachment, invasion, and establishment of the intracellular parasitophorous vacuole, within which parasite replication takes place. Remarkably, these organisms harbor a secondary endosymbiotic organelle, acquired when an ancient protist engulfed (or was invaded by) a eukaryotic alga -- and retained the algal plastid. The 'apicoplast' is an essential organelle, and a promising target for antiparasitic drug development. Studies on the

apicoplast also reveal much about the modular nature of eukaryotic targeting signals, and the evolution of endosymbiotic organelles. The availability of complete genome sequence for numerous apicoplexan parasites enables comparative genomic analysis, and robust systems for molecular genetic manipulation of *T. gondii* provide transgenic parasites for exploring host immune responses.

## Building Sensory Networks (1472-1477)

1472

### Dissecting the Molecular Basis of Photoreceptor Target Selection in the *Drosophila* Visual System

T. R. Clandinin; Neurobiology, Stanford University, Stanford, CA

Understanding how the complex wiring diagram of the central nervous system emerges during development is one of the central challenges in neurobiology. Our work is focused on characterizing the molecular mechanisms that allow neurons to choose specific synaptic partners, using the connections between photoreceptor cells and their primary targets in the Drosophila visual system as a model. Photoreceptor cells that look at the same point in space converge on a common set of target neurons. Remarkably, these precise connections are genetically hard-wired, emerging in the absence of visual input. Using a genetic screen based on a specific visual behavior, we have identified three cell surface molecules, namely two cadherin family members and one receptor tyrosine phosphatase, as playing critical roles in photoreceptor targeting. We are currently characterizing the genetic and biochemical interactions between these molecules. Ongoing experiments have identified additional molecular components. These experiments are likely to define a number of general molecular mechanisms that allow developing neurons to make choices between alternate synaptic partners in a wide range of contexts and animals.

1473

### The Role of Axon-Axon Competition in Retinotectal Map Formation

N. J. Gosse,[1] L. M. Nevin,[2] H. Baier[1,2,3]; [1]Program in Genetics, University of California, San Francisco, San Francisco, CA, [2]Program in Neuroscience, University of California, San Francisco, San Francisco, CA, [3]Department of Physiology, University of California, San Francisco, San Francisco, CA

We have devised a new experimental paradigm for the study of visual map development. In the zebrafish mutant *lakritz*, no retinal ganglion cells (RGCs) are formed, due to a null mutation in the bHLH transcription factor Ath5 (Atoh7). The *lakritz* tectum is therefore devoid of retinal input, but is otherwise normal, as judged by its size, gene expression, and cell-type composition. Wildtype (wt) cells grafted into a *lakritz* host at the blastula stage may give rise, later in development, to a chimera with small clones of wt cells in an otherwise mutant retina. These clones often contain just one RGC, which in turn projects a single axon into the tectum. This configuration has now allowed us to address the role of axon-axon interactions in retinotectal development. We first asked whether a solitary RGC axon would terminate in a topographically accurate location in the tectum, i.e. at the anterior-posterior position predicted by its nasal-temporal origin in the retina. We found that retinal axons in wt->*lakritz* chimeras terminated at approximately the same position as retinal axons in wt->wt chimeras. When all data were combined into a graph plotting tectal arbor position over retinal origin, a smooth map was observed with a slight anterior shift for the solitary axons over the crowded condition. Synaptic competition between neighboring retinal axons has been put forward as a mechanism to restrict arbor size in the tectum. Indeed, we observed that the arbors of solitary axons were twice as large and more complex than in wildtype. We conclude that axon-axon competition plays a surprisingly small role in retinotopic targeting, but strongly influences the elaboration of retinal arbors in the tectum.

1474

### Intraflagellar Transport (IFT) is Required for the Vectorial Movement of TRPV Channels in the Ciliary Membrane

H. Qin,[1] D. T. Burnette,[1] Y. Bae,[2] P. Forscher,[1] M. M. Barr,[2] J. L. Rosenbaum[1]; [1]MCDB Dept., Yale University, New Haven, CT, [2]School of Pharmacy, University of Wisconsin, Madison, WI

The membranes of all eukaryotic motile (9 +2) and immotile primary (9 +0) cilia harbor channels and receptors involved in sensory transduction. These membrane proteins are transported from the cytoplasm onto the ciliary membrane by vesicles targeted for exocytosis at a point adjacent to the ciliary basal body. Here, we use time-lapse fluorescence microscopy and genetics to demonstrate that select GFP-tagged sensory receptors undergo rapid vectorial transport along the entire length of the cilia of *Caenorhabditis elegans* sensory neurons in vivo. Transient receptor potential vanilloid (TRPV) channels OSM-9 and OCR-2 move in ciliary membranes at rates comparable to the intraflagellar transport (IFT) machinery located between the membrane and the underlying axonemal microtubules. OSM-9 and OCR-2 motility is disrupted in certain IFT mutant backgrounds. Surprisingly, motility of transient receptor potential polycystin (TRPP) channel PKD-2, a mechano-receptor, is not detected. Our study demonstrates that IFT, previously shown to be necessary for transport of axonemal components, is also required for the motility of TRPV membrane protein movement along cilia of *C. elegans* sensory cells.

1475

### Neural Ensemble Codes in Fly Olfaction

G. Miesenboeck; Department of Cell Biology, Yale University School of Medicine, New Haven, CT

To understand how information is represented by neuronal assemblies, we study the olfactory system of the fly, Drosophila melanogaster. Our experimental approach relies on genetically encoded optical sensors capable of revealing the activities of distinct neuronal populations in the living brain. Representations of odors and odor blends can thus be visualized in virtual isolation at successive, synaptically coupled stages of intact processing cascades, and the nature of the neural operations performed at and between stages can be deduced.

1476

### Dissecting Neural Networks Using Targeted Cell Class Ablation

S. Nirenberg; Dept. of Neurobiology, UCLA, Los Angeles, CA

Our research focuses on the general question "How do neural networks carry out computations? While a great deal is known about individual cells in the nervous system - in terms of their physiological properties and their patterns of connections - relatively little is known about how they act together in networks to produce specific outputs or carry out computations. Even computations that seem relatively simple, such as determining the

direction of motion of a moving object or the orientation of a line are still not completely understood. One approach to this problem is to dissect the networks using targeted cell class ablation - that is, dissect them by eliminating different classes of cells and evaluating how the network output is perturbed. The focus of my talk will be on a method we developed for this purpose and the results of using it to study network function.

1477

**Enhanced Signaling of a Serotonergic Circuit Regulates Aversive Olfactory Learning of *C elegans***

Y. Zhang, H. Lu, C. I. Bargmann; HHMI, The Rockefeller University, New York, NY

An animal's ability to avoid harmful food is essential for its survival. One robust form of olfactory learning is conditioned avoidance of food associated with noxious stimuli. In its natural soil habitat, *C. elegans* feeds on bacteria, potentially including pathogenic bacteria. Common soil bacteria *Pseudomonas aeruginosa* and *Serratia marcescens* can proliferate in *C. elegans'* intestine and cause death[1]. Learning to avoid pathogens could promote *C. elegans'* survival. Using an olfactory choice assay we found that animals raised on *S. marcescens* or *P. aeruginosa* supplemented with *E. coli* OP50 (standard worm food) avoided the pathogen when it was presented in a choice with OP50, while animals raised on OP50 showed no preference. This experience-based olfactory change is distinct from adaptation and appears to represent a form of associative learning similar to taste aversion. Adult animals exposed to pathogen acquire an aversion within a few hours, indicating that olfactory learning is an acute modification of animals' innate preferences. Different pathogens induce olfactory changes specific to animal's experiences. Using a multiple-choice assay, we found that trained animals acquired both an aversion to the training pathogen and an increased attraction for OP50. To identify modulatory pathways in pathogen-learning, we tested *tph-1* mutants, which are defective in serotonin synthesis. *tph-1* animals are deficient in both attractive and aversive learning. Increased serotonin contents are detected by HPLC in pathogen-trained animals; enhanced serotonin immunoreactivity is specifically detected in serotonergic neurons, ADF. Expression of *tph-1* cDNA in ADF fully rescues aversive learning. We also identified MOD-1, a serotonin-gated chloride channel, functioning in interneurons downstream of ADF to promote aversive learning. We concluded that pathogen exposure enhances serotonergic signaling in ADF neurons, stimulating aversive learning through MOD-1. (1). J.J. Ewbank, *Microbes Infect* 4, 247-56.

## Coordinating Adhesion & Signaling (1478-1483)

1478

**Coordinating Adhesion and Signaling in Colon Cancer Cells by Novel Target Genes of Beta-Catenin-Cadherin Signaling**

A. Ben-Ze'ev,[1] N. Gavert,[1] M. Conacci-Sorrell,[1] P. Altevogt,[2] T. Brabletz[3]; [1]Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel, [2]Tumor Immunology Programme, German Cancer Research Center, Heidelberg, Germany, [3]Department of Pathology, University of Erlangen-Nurenberg, Erlangen, Germany

Aberrant β-catenin signaling is prevalent in most colorectal cancer patients. β-catenin, an important cell-cell adhesion component binding cadherin receptors to the cytoskeleton, is also a key co-transcriptional activator of target genes (with LEF/TCF factors) in the nucleus. Hyperactive β-catenin induces genes regulating the cell cycle and the invasion and metastasis of cancer cells. Using human colon cancer cells, we found that sparse cultures mimic cells at the invasive front of tumors displaying low E-cadherin levels, but highly active nuclear β-catenin that transactivates *Slug*, a negative transcriptional regulator of E-cadherin. These cells also expressed abundant ErbB1/2 and active ERK. In contrast, dense cultures had distinct membranal E-cadherin and β-catenin, only limited β-catenin signaling, no Slug, and therefore high E-cadherin levels, but low levels of ErbB1/2 and MAPK signaling. This cell density-regulated phenotypic conversion is reminiscent of the plasticity in β-catenin and E-cadherin in the invasive versus the differentiated colorectal carcinoma tissue. We identified two members of the L1 cell adhesion family (normally expressed in nerve cells), as novel target genes of β-catenin, and found them exclusively in the invasive front of colon carcinoma tissue. Forced expression of L1 and Nr-CAM in fibroblasts induced motility, transformation, conferred tumorigenicity in mice, and liver metastasis in colon cancer cells. Suppression of L1 in colon cancer cells reduced their motility and ECM invasion. We suggest that these "neuronal" adhesion molecules, when aberrantly activated by β-catenin, are exploited opportunistically by colon cancer cells to promote metastasis. Since the extracellular domains of L1 and Nr-CAM are often shedded by metalloproteinases, they could serve as diagnostic markers and anticancer therapy targets. We identified L1 and the metalloproteinase ADAM10 at the invasive front of human colorectal cancer tissue.

1479

**A Central Role for p120ctn in the Regulation of Contact Inhibition of Cell Growth**

G. A. Wildenberg, R. Carnahan, M. Dohn, A. B. Reynolds; Cancer Biology, Vanderbilt University, Nashville, TN

p120ctn binds directly to the juxtamembrane domain of classical cadherins and is essential for proper cadherin function and stability. Overexpression of p120 in fibroblasts induces dramatic branching of cellular processes, apparently due to inhibition of the small GTPase RhoA. Here, we show that siRNA-based p120 ablation in NIH3T3 cells induces constitutive activation of RhoA and a dramatic increase in actin stress fibers. Interestingly, key branches of receptor tyrosine kinase and integrin signaling pathways that regulate the cytoskeleton through inhibition of RhoA are completely blocked, and the cells exhibit cancer-related phenotypes such as focus formation, proliferation in the absence of serum, and loss of contact-mediated inhibition of cell growth. We have found that the failure of contact inhibition occurs because p120 is an essential link in a signaling pathway that allows cadherin-ligation to regulate cell cycle progression. Our data reveal that p120 functions as a potent negative regulator of RhoA via dynamic assembly of novel p120-linked complexes, and these complexes mediate contact inhibition of cell growth.

1480

**A New Role for Desmoplakin in Intracellular Signaling?**

A. P. Miera, I. Gallicano; Cell Biology, Georgetown University, Washington, DC

For 30 years the canonical function for desmoplakin has been to link intermediate filaments to desmosomes. However, recent evidence strongly suggests that desmoplakin may also have nonconical functions. An example is the recent discovery of DP in capillaries and lymphatic vessels. DP has also been linked to the cell adhesion molecule, PECAM, which has a role in adhesion and signal transduction. In fact, the relationship between PECAM and DP led us to investigate if DP may have a role in cell signaling. To do so, we employed a high-throughput proteomics assay whereby

Luciferase-tagged DP cDNA bait was coexpressed with over 500 Flag-tagged prey cDNAs, followed by immunoprecipitation with a flag antibody and detection using the luciferase assay. Using this high throughput assay, we found three guanine exchange factors (GEFs) directly associated with DP. We analyzed the effects caused by GEF/DP interaction focusing on RhoA activity and its downstream targets in human embryonic keratinocytes. Co-immunoprecipitation studies revealed that GEF-1 directly associates with DP in a spatio-temporal manner during early time points of terminal differentiation. Inhibition of this binding by knocking down GEF1 resulted in diminished desmosome formation and diminished RhoA activity. Knocking down DP prior to inducing terminal differentiation and desmosome formation also resulted in diminished RhoA activity, which was restored upon DP siRNA degradation and re-expression of DP protein. Finally, using a fluorescent reporter that measures RhoA activity, we temporally and spatially confirmed the GEF/DP dependent-activation of RhoA at cell junctions during differentiation. Consequently, GEF/DP interaction is needed for normal RhoA activity in keratinocyte differentiation.

1481

**Actin Remodelling during Epithelial Polarization: Distinct Actin Populations and Signalling Events**

V. M. M. Braga,[1] J. K. Zhang,[1] M. E. Betson,[1] J. Erasmus,[1] K. Zeikos,[1] M. Bailly,[2] L. Cramer[3]; [1]Division of Biomedical Sciences, Faculty of Life Sciences, Imperial College London, London, United Kingdom, [2]Institute of Ophthalmology, Imperial College London, London, United Kingdom, [3]MRC-LMCB, Imperial College London, London, United Kingdom

During establishment of cell-cell contacts, a tight coordination between cell adhesion and cytoskeletal remodelling is essential to ensure the morphology and integrity of epithelial sheets. These changes in cytoarchitecture are accompanied by the acquisition of functional and signalling properties that are intrinsic to epithelial function**.** The organization of actin filaments in polarized epithelia has been known for many years. However, during establishment of cell-cell adhesion, the temporal and spatial changes in actin dynamics and remodelling are poorly understood. During keratinocyte polarization, we identified two distinct actin pools: junctional-actin and thin actin bundles. These two actin populations differ in their actin dynamics, mechanism of formation and interestingly, have distinct roles during epithelial polarization. While junctional-actin stabilizes clustered cadherin receptors at cell-cell contacts, peripheral actin bundle reorganization is essential for increase in cell height during polarization. The contribution of Rho GTPases to the formation of each actin population will be discussed in a model that integrates the adhesion-dependent changes in actin dynamics and specific signalling pathways to form a polarized cell shape.

1482

**E-cadherin Engagement Stimulates Proliferation via Rac1**

W. F. Liu,[1] C. M. Nelson,[1] D. M. Pirone,[2] C. S. Chen[2]; [1]Biomedical Engineering, Johns Hopkins University, Baltimore, MD, [2]Bioengineering, University of Pennsylvania, Philadelphia, PA

E-cadherin has been linked to suppression of tumor growth and contact inhibition of cell proliferation in culture, but is also present in tissues where cells continuously proliferate. The mechanism by which E-cadherin regulates proliferation is complex and remains elusive. Here we observed that progressively decreasing seeding density of NRK-52E or MCF10A from maximal confluence released cells from growth arrest, reaching the highest levels of proliferation at intermediate densities. Unexpectedly, further decreasing seeding density so that cells were isolated from their neighbors then decreased proliferation, suggesting that cell-cell contacts were responsible for the increased proliferation rates observed only at intermediate densities. Introducing cell-cell contacts by either increasing the number of cells within multicellular aggregates in three-dimensional cultures, or by examining cells in microwell cultures, also suggested increased proliferation when limited cell-cell contact occurred. The peak level of proliferation observed at intermediate densities was abrogated by expression of a dominant negative mutant of E- cadherin or exposure to blocking antibodies. Rac1 activity and associated lamellepodial ruffling and extension increased with cell-cell contact to maximal levels also at intermediate densities, and were abrogated with expression of dominant negative E-cadherin. Furthermore, inhibition of Rac1 signaling by expression of dominant negative mutants blocked E- cadherin-induced proliferation. The projected area of cell spreading against the underlying substrate correlated directly with proliferation, with highest degrees of spreading at intermediate cell densities, however microfabricated microwell cultures demonstrated that hese changes in cell spreading were not required for the cell-cell contact- induced increases in proliferation. In sum, these findings offer a simple mechanism by which cell-cell contact may stimulate or inhibit proliferation depending on the degree of contact, and demonstrate that dynamic changes in E-cadherin engagement may be used in different settings to trigger different proliferative responses.

1483

**Mechanical Force Mobilizes Zyxin from Focal Adhesions to Actin Filaments and Regulates Cytoskeletal Reinforcement**

M. Yoshigi, L. M. Hoffman, C. C. Jensen, H. Yost, M. C. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Organs and tissues adapt to acute or chronic mechanical stress through remodeling their actin cytoskeletons. Cells stimulated by cyclic stretch or shear stress in vitro undergo bi-modal cytoskeletal responses that include rapid reinforcement and gradual reorientation of actin stress fibers, however the mechanism by which cells respond to mechanical cues has been obscure. We report here that application of either unidirectional cyclic stretch or shear stress to cells results in robust mobilization of zyxin from focal adhesions to actin filaments while many other focal adhesion proteins and zyxin family members remain at focal adhesions. The mobilization of zyxin from focal adhesions does not occur in stretched cells plated on poly-lysine, suggesting that integrin-dependent adhesion is required for the response. The redistribution of zyxin upon stretch is preserved in cells treated with gadolinium, thus the response appears not to rely on stretch-activated channels. Mechanical stress also induces rapid, zyxin-dependent mobilization of Vasodilator-Stimulated Phosphoprotein (VASP) from focal adhesions to actin filaments. Thickening of actin stress fibers reflects a cellular adaptation to mechanical stress; this cytoskeletal reinforcement occurs coincident with zyxin mobilization, is abrogated in zyxin-null cells, and is restored upon re-expression of zyxin. Interestingly, the reorientation of cytoskeletal elements in response to uniaxial stretch occurs normally in zyxin-null cells, thus demonstrating that the cytoskeletal reinforcement and reorientation responses are mechanistically distinct. Our findings identify zyxin as a mechanosensitive protein and provide mechanistic insight into how cells respond to mechanical cues.

## Cytoskeletal Molecular Motors (1484-1489)

1484

### Dimerization, but not the Dynein Associated Proteins, is Required for Processive Movement of Yeast Cytoplasmic Dynein

S. L. Reck-Peterson, A. P. Carter, A. Yildiz, A. Gennerich, R. D. Vale; Cellular and Molecular Pharmacology, UCSF, San Francisco, CA

Cytoplasmic dynein is responsible for nearly all minus-end directed microtubule based traffic in most eukaryotic cells and has additional roles in mitosis; however, its mechanism of movement is poorly understood compared to kinesin and myosin. We have developed a method to purify native cytoplasmic dynein and a minimal functional motor domain from *S. cerevisiae.* Using total internal reflection and optical trap microscopy, we find that yeast cytoplasmic dynein is a highly processive motor with a run length of ~8 um and a stall force of ~6 pN. Cytoplasmic dynein processivity requires a dimer of two motor domains. As evidence, we find that monomeric dynein is not processive, but can be induced to move processively by artificial dimerization. By using yeast strains deleted for various dynein associated proteins, we show that the dynein heavy chain is sufficient for processivity, although associated proteins may affect the run length. Finally, to address the stepping mechanism of this motor, we have performed FIONA (Fluorescence Imaging with One Nanometer Accuracy) experiments with dynein molecules labeled on a single motor domain or on the tail domain and have been able to resolve individual steps at low ATP concentration. Based on this data, we propose a model in which dynein moves by a hand-over-hand mechanism with a primary displacement of ~8 nm per cycle although occasional larger steps are observed. Thus, like kinesin and myosin V, cytoplasmic dynein moves processively through the coordination of its two motor domains.

1485

### Myosin VI is a Dimer Due to Actin *in vivo*

H. Park,[1] H. Lee Sweeney,[2] P. R. Selvin[1]; [1]Department of Physics and Biophysics, University of Illinois, Urbana-Champaign, Urbana, IL, [2]Penn. Muscle Institute, University of Pennsylvania, Philadelphia, PA

Myosin VI is a reverse direction actin-based motor capable of taking surprisingly large steps (~30 nm center-of-mass; 60 nm hand-over-hand) when dimerized. However, all dimeric myosin VI molecules that have been examined to date have included a non-native coiled-coil sequences. Reports on full length myosin VI have failed to demonstrate the existence of dimers, and it has been suggested that the predicted coiled-coil sequence within myosin VI may not be capable of dimerization. Here we demonstrate that full-length myosin VI is capable of forming a stable, processive dimer when it binds to actin, moving distances up to 1.1 µm with approximately 30 nm center-of-mass steps in a hand-over-hand fashion. Upon shortening by 203 a.a., removing the cargo-binding domain, the molecule is still processive, with a large fraction (90%) of molecules moving. Upon further shortening by a total of 336 a.a.'s, removing much of the coiled-coil domain, very few myosin VI molecules move, and removing the whole coiled-coil domain makes it non-processive. Furthermore, we present data consistent with the molecule being in an equilibrium between folded and unfolded monomers that are prevented from dimerizing by an inhibitory region within both the motor domains. A model thus emerges that cargo binding likely clusters full length myosin VI molecules, but actin binding is required to induce dimerization by inhibitory regions in both the motor domain and the cargo-binding domain.

1486

### Structural Analysis of Myosin-V Discrimination between Distinct Cargoes

N. Pashkova,[1] Y. Jin,[2] S. Ramaswamy,[1] L. S. Weisman[2]; [1]Biochemistry, University of Iowa, Iowa City, IA, [2]Life Sciences Institute, University of Michigan, Ann Arbor, MI

Myosin V molecular motors function in relatively long-range movement on actin filaments. In a single cell type, a myosin V may move multiple cargoes to distinct places at different times. Indeed the *Saccharomyces cerevisiae* myosin V, Myo2p, moves multiple cargoes including secretory vesicles, the late Golgi, peroxisomes, vacuoles, and the nuclear spindle. The identification of specific mutations that affect the movement of a single cargo has suggested that each cargo binds a distinct region on myosin V. Here we report the crystal structure at 2.2 Å of the myosin V cargo-binding domain. It is comprised of two helical bundles. There are several patches of highly conserved regions distributed on the surface which are candidate attachment sites for cargo-specific receptors. We identified a region of conserved surface residues that is solely required for vacuole inheritance and a second region that is required for secretory vesicle movement, but not vacuole movement. These cargo-binding regions are at opposite ends of the oblong-shaped cargo-binding domain, and moreover are offset by approximately 180° . This large separation of cargo-binding areas strongly suggests that the globular tail of myosin V serves as a scaffold for binding multiple cargoes. That both the vacuole binding region and secretory vesicle region are simultaneously exposed on the surface of the globular tail, suggests that the major targets for the regulation of cargo movement are organelle-specific myosin V receptors.

1487

### Cik1 Targets the Minus-end Kinesin Depolymerase Kar3 to Microtubule Plus-ends

L. R. Sproul,[1] D. J. Anderson,[1] A. T. Mackey,[2] W. S. Saunders,[1] S. P. Gilbert[1]; [1]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, [2]Department of Molecular, Cellular, and Developmental biology, Yale University, New Haven, CT

Kar3, a *Saccharomyces cerevisiae* Kinesin-14, is essential for karyogamy and meiosis I, but also has specific functions during vegetative growth. For its various roles during the yeast life cycle, Kar3 forms a heterodimer with either Cik1 or Vik1, both of which are non-catalytic polypeptides. We will present the first biochemical characterization of Kar3Cik1, the kinesin motor which is essential for karyogamy. Kar3Cik1 depolymerizes microtubules from the plus-end and promotes robust minus-end directed microtubule gliding (2.4 µm/min). Immunolocalization studies show that Kar3Cik1 binds preferentially to one end of the microtubule, while the Kar3 motor domain in the absence of Cik1 exhibits significantly higher microtubule lattice binding. Kar3Cik1-promoted microtubule depolymerization requires ATP turnover, and the kinetics fit a single exponential function ($k_{obs}$ = 0.07 $s^{-1}$). The disassembly mechanism is not microtubule catastrophe like that induced by the MCAK Kinesin-13s. Soluble tubulin does not activate the ATPase activity of Kar3Cik1, and there is no evidence of Kar3Cik1-tubulin complex formation as observed for MCAK. These results reveal a novel mechanism to regulate microtubule depolymerization. We propose that Cik1 targets Kar3 to the microtubule plus-end. Kar3Cik1 then uses its minus-end directed force to depolymerize microtubules from the plus-end with each tubulin subunit release event tightly coupled to one ATP turnover. Supported by NIH GM54141 and K02-AR47841 to SPG.

1488

**Self-assembly is Important for the Function of Centralspindlin, a Protein Complex of Mitotic Kinesin and RhoGAP**

M. Mishima,[1,2] M. Glotzer[3,2]; [1]Wellcome/CRUK Gurdon Institute, University of Cambridge, Cambridge, United Kingdom, [2]Research Institute of Molecular Pathology (IMP), Vienna, Austria, [3]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

Cytokinesis is essential for cell proliferation and its accurate execution is important for the maintenance of genomic stability. Antiparallel microtubule structures play critical roles in cytokinesis. The central spindle, together with astral microtubules, contributes to specification and promotion of cleavage furrow ingression. The midbody is indispensable for the completion of cytokinesis. Proper assembly of these microtubule structures is dependent on centralspindlin, an evolutionarily conserved protein complex consisting of ZEN-4/Pavarotti/MKLP1 kinesin and CYK-4/RacGAP50C/MgcRacGAP RhoGAP, which has microtubule-bundling activity. It accumulates on the central spindle during anaphase and highly concentrates to the center of the midbody during telophase. The mechanism by which centralspindlin accumulates exclusively on the central spindle and later to the center of the midbody, and not to other microtubule structures, is not known. We have found that centralspindlin undergoes self-assembly into higher order structures. The self-assembly is mediated by a defined region in the putative coiled coil of ZEN-4, the mitotic kinesin subunit in *C. elegans*. A centralspindlin complex whose ZEN-4 subunit is deficient in self-assembly is defective in microtubule bundling. A self-assembly-deficient zen-4 transgene rescued zen-4 null animals less efficiently than wild type zen-4 transgene. These results suggest an important role of self-assembly in the function of centralspindlin.

1489

**Myosin VII and Cell Adhesion**

M. A. Titus, S. A. Galdeen, S. Stephens; Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

The class VII myosins (M7s) are characterized by the presence of two MyTH4/FERM domains in their tail and are widely expressed throughout phylogeny. Dictyostelium cells lacking M7 are defective in adhesion to substratra and filopod extension. The major Dictyostelium M7 binding partner is talinA, a FERM domain protein with a role in adhesion. TalinA levels are directly linked to M7 levels - overexpression of M7 (either the full-length protein or the tail) results in increased talinA levels and cells lacking M7 have decreased talinA levels. Both proteins are localized to the plasma membrane of Dictyostelium cells, and enriched where actin-based protrusions such as lamellipodia are extended. Deletion studies reveal that removal of a single FERM domain from the M7 tail does not alter localization to the plasma membrane but that deletion of both FERM domains abolishes M7 membrane localization. Two FERM domains linked together, either with or without their preceding MyTH4 domains, fail to localize to the cortex indicating that the FERM domains are necessary but not sufficient for targeting M7 to the plasma membrane. While talinA is not required for M7 localization to the plasma membrane, preliminary IP results reveal additional M7 binding partners in a membrane-cytoskeleton fraction that may play a role in the cortical localization of M7. The role of M7 in adhesion is also being explored in C. elegans. A mutant carrying a deletion in the hum-6 gene encoding the sole M7 is embryonic lethal. The embryos die at the two-fold stage, a time when hypodermis-body wall muscle attachment is critical for continued development, suggesting that M7 may also play a role in cell-cell adhesion in worms.

## Intermediate Filaments (1490-1495)

1490

**A Dominant Vimentin Mutation Causes a Posterior Cataract in Mice**

R. Meier-Bornheim,[1] H. Büssow,[2] D. Bröhl,[2] S. Loch,[1] T. M. Magin[1]; [1]Physiological Chemistry, University of Bonn, Bonn, Germany, [2]Division of Cellular Biochemistry and LIMES, University of Bonn, Bonn, Germany

To gain new insights into the function of the intermediate filament (IF) protein vimentin *in vivo*, we generated transgenic mice which express a mutant vimentin gene controlled by its promoter. The mutation was designed to cause a mutation in the consensus motif in coil1A ($R_{113}C$). Analogous mutations in keratins and GFAP lead to keratinopathies and Alexander disease, respectively. In transgenic mice, $R_{113}C$ acted in a dominant-negative way and displayed cytoplasmic vimentin aggregates in all tissues examined. Vimentin inclusions were most prominent in lens fibre cells and began to form a few days after birth. In due course, lens fibre cells became highly disorganised and were disrupted, culminating in a posterior capsule rupture. Vim$R_{113}C$-expressing lens fibre cells showed premature enucleation, suggesting an involvement of vimentin in this process. Moreover, the mutant caused a strong upregulation of hsp70 but did not interfere with the expression and organisation of the specific lens IF proteins filensin and phakinin. The center of the lens became devoid of aggregated vimentin, demonstrating that aggregates, even in enucleated fibre cells, can be degraded. This was mediated by an upregulation of proteasome activity, which in addition may contribute to lens pathology. In addition, we demonstrated an upregulation of GFAP in the lens epithelium of vim$R_{113}C$-mutant mice, suggesting that the mutant induced a tissue repair process in the regenerating epithelium. Upon expression in cultured fibroblasts, vim$R_{113}C$ induced an increase in the stress-activated kinases ERK1/2. Collectively, our data suggest that vimentin mutations may cause cataracts in humans.

1491

**Novel Roles of Intermediate Filaments in Adhesion and Transcellular Migration**

M. Nieminen,[1] T. Henttinen,[1] M. Merinen,[2] F. Marttila,[2] S. Jalkanen,[2] J. E. Eriksson[1]; [1]Dept. of Biology, University of Turku, Turku, Finland, [2]MediCity Research Unit, University of Turku, Turku, Finland

While the adhesive interactions of leukocytes with endothelial cells are well established, little is known about the detailed mechanisms underlying the actual diapedesis of leukocytes. We have observed that the transmigration commences through two different routes. While polymorphonuclear (PMN) cells (mainly neutrophils) migrate between endothelial cells through junctional areas (paracellular pathway), strikingly peripheral blood mononuclear cells (PBMCs; T and B lymphocytes) were able to pass straight through the endothelial cell (EC) body (transcellular route). The endothelial and the lymphocyte intermediate filaments (IFs) formed a highly dynamic anchoring structure at the site of the EC-PBMC contact. The initiation of this process was markedly reduced in vimentin-deficient (vim -/-) ECs and PBMCs. Furthermore, when compared to wildtype PBMCs, vim -/- PBMCs showed markedly reduced capacity to home to mesenteric lymph nodes and spleen when injected into the mice. These results show that IFs play an active role during lymphocyte adhesion and transmigration, which is a completely novel function for IFs. The dynamic IF structure provides a molecular basis for the transmigratory traction assembly of adhesion molecules. In support of this hypothesis, vim -/- ECs and PMBCs

both showed highly aberrant expression and distribution of surface molecules critical for homing (ICAM-1 and integrin- 1). The molecular mechanisms of IF-adhesion molecule interaction and the role of phosphorylation will be discussed.

323A

**KIF5A is Required for Both Anterograde and Retrograde Movement of Neurofilaments in Axons**

A. Uchida, A. Brown; Center for Molecular Neurobiology, The Ohio State University, Columbus, OH

Neurofilaments are transported bidirectionally along axons in a rapid infrequent manner. The retrograde motor is dynein but the anterograde motor is unknown. Recently the Goldstein lab reported that targeted deletion of KIF5A in mice causes neurofilament accumulation in DRG cell bodies and a reduction in axonal caliber with no apparent effect on organelle transport, suggesting that KIF5A is an anterograde neurofilament motor. To test this hypothesis, we cultured neurons from wild type (WT) and KIF5A-/- mice. Both sympathetic (SCG) and sensory (DRG) KIF5A-/- neurons extended axons that contained neurofilaments. To examine neurofilament transport, we performed time-lapse fluorescence imaging on SCG neurons expressing GFP-tagged neurofilament protein. The frequency of both anterograde and retrograde neurofilament movement was reduced significantly compared to WT ($p<0.001$, K-S test; average reduction=75%). For those neurofilaments that moved, the average anterograde velocity (excluding pauses) was reduced significantly ($p=0.001$, K-S test; average reduction=52%) but the average retrograde velocity was not ($p=0.4$, K-S test). Immunostaining revealed apparently normal dynein/dynactin levels in KIF5A-/- axons and Western blotting revealed normal levels of dynein/dynactin and kinesin light chains in KIF5A-/- brain. Expression of full-length KIF5A in KIF5A-/- SCG neurons rescued the frequency of neurofilament movement in both directions and partially rescued the anterograde velocity. Expression of a “dominant negative” headless KIF5A in WT SCG neurons completely inhibited movement in both directions. Collectively these data indicate that (1) KIF5A is the principal but not exclusive anterograde motor for neurofilaments in SCG neurons, (2) proteins with homology to KIF5A, probably KIF5B and/or KIF5C, may also be capable of moving neurofilaments anterogradely, and (3) KIF5A is necessary for normal retrograde movement of neurofilaments, which suggests a direct or indirect interaction between the dynein and kinesin motors.

1492

**KIF5A is Required for Both Anterograde and Retrograde Movement of Neurofilaments in Axons**

A. Uchida, A. Brown; Center for Molecular Neurobiology, The Ohio State University, Columbus, OH

Neurofilaments are transported bidirectionally along axons in a rapid infrequent manner. The retrograde motor is dynein but the anterograde motor is unknown. Recently the Goldstein lab reported that targeted deletion of KIF5A in mice causes neurofilament accumulation in DRG cell bodies and a reduction in axonal caliber with no apparent effect on organelle transport, suggesting that KIF5A is an anterograde neurofilament motor. To test this hypothesis, we cultured neurons from wild type (WT) and KIF5A-/- mice. Both sympathetic (SCG) and sensory (DRG) KIF5A-/- neurons extended axons that contained neurofilaments. To examine neurofilament transport, we performed time-lapse fluorescence imaging on SCG neurons expressing GFP-tagged neurofilament protein. The frequency of both anterograde and retrograde neurofilament movement was reduced significantly compared to WT ($p<0.001$, K-S test; average reduction=75%). For those neurofilaments that moved, the average anterograde velocity (excluding pauses) was reduced significantly ($p=0.001$, K-S test; average reduction=52%) but the average retrograde velocity was not ($p=0.4$, K-S test). Immunostaining revealed apparently normal dynein/dynactin levels in KIF5A-/- axons and Western blotting revealed normal levels of dynein/dynactin and kinesin light chains in KIF5A-/- brain. Expression of full-length KIF5A in KIF5A-/- SCG neurons rescued the frequency of neurofilament movement in both directions and partially rescued the anterograde velocity. Expression of a “dominant negative” headless KIF5A in WT SCG neurons completely inhibited movement in both directions. Collectively these data indicate that (1) KIF5A is the principal but not exclusive anterograde motor for neurofilaments in SCG neurons, (2) proteins with homology to KIF5A, probably KIF5B and/or KIF5C, may also be capable of moving neurofilaments anterogradely, and (3) KIF5A is necessary for normal retrograde movement of neurofilaments, which suggests a direct or indirect interaction between the dynein and kinesin motors.

1493

**Interaction of Keratin 17 with 14-3-3 Regulates Protein Synthesis and Cell Growth**

S. Kim, P. Wong, P. A. Coulombe; Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD

Upregulation of keratin 17 (K17), a type I intermediate filament protein, is a hallmark of stratified epithelia undergoing repair, but its role in this setting has yet to be defined. Here we show that this abundant cytoskeletal protein regulates protein synthesis and epithelial cell growth through its phosphorylation-dependent binding to 14-3-3sigma (stratifin), a multifunctional adaptor protein. Mouse keratinocytes lacking K17 exhibit depressed protein translation and a smaller size correlating with a decrease in the activity of the Akt/mammalian target of rapamycin (mTOR) pathway. Binding of K17 to 14-3-3 is required for the serum-dependent export of 14-3-3 from the nucleus to the cytoplasm, and subsequent stimulation of mTOR activity and protein synthesis. Loss of this function likely accounts for the delay in ectodermal wound closure exhibited by K17 null embryos, a physiological context in which lack of this keratin is associated with a smaller cell size. These findings reveal a novel and unexpected role whereby the intermediate filament cytoskeleton influences cell growth and size by regulating protein synthesis.

1494

**Keratin-8 Mutation Predisposes to Liver Injury by Altering Stress Kinase-mediated Phosphorylation**

N. Ku, M. B. Omary; Medicine, Palo Alto VA Medical Center and Stanford University, Palo Alto, CA

Keratin 8 (K8) G61C mutation is a risk factor for developing end-stage liver disease, as determined by human association studies, but direct evidence for predisposition to liver injury via a natural human K8 mutation is lacking. To investigate the in vivo significance of the human liver disease-associated K8 G61C mutation, we generated transgenic mice that overexpress wild type (WT) or G61C human (h) K8 and compared their susceptibility to stress-induced liver injury. K8 expression was comparable in WT and G61C K8 lines, and there was no significant effect of hK8 transgene expression on endogenous keratin filament organization or on baseline liver histology. However, K8 G61C mice are significantly more susceptible to Fas- or microcystin-LR-induced liver injury as compared to non-transgenic and WT K8 animals. Most deaths occurred within 6-12 hr after drug administration, and histologic assessment after injury supported the mortality data. In mice and transfected cells, K8 G61C decreases dramatically K8 Ser73 phosphorylation by stress-related kinases. K8 G61C is highly insoluble and generates cross-linked keratins in transgenic

mouse livers after administration of Fas or the oxidizing agent paraquat, which may contribute to kinase inaccessibility to K8 Ser73. The importance of K8 Ser73 phosphorylation in protection from liver injury was confirmed by generating mice that overexpress K8 S73A which manifest increased susceptibility to injury similar to G61C mice. These findings provide direct evidence that naturally occurring human K8 mutations predispose to liver injury, and suggest an important role for K8 in protecting hepatocytes from injury by serving as a phosphate sink for stress-activated kinases. This role may extend to keratin-related diseases in other tissues where the K8 S73-containing epitope is conserved.

1495

**The Common Lamin A (LA) Mutation in Hutchison Gilford Progeria (HGPS) Causes a Decrease in Histone Methylation**

D. K. Shumaker,[1] T. Dechat,[1] M. R. Bozovsky,[1] A. E. Goldman,[1] S. Khuon,[1] S. A. Adam,[1] A. Kohlmaier,[2] M. R. Erdos,[3] M. Eriksson,[4] F. S. Collins,[3] T. Jenuwein,[2] R. D. Goldman[1]; [1]Cell Molecular Biology, Northwestern University The Feinberg School of Medcine, Chicago, IL, [2]Research Institute of Molecular Pathology, Vienna, Austria, [3]Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, [4]Department of Medical Nutrition, Karolinska Institutet, Huddinge, Sweden

There is a loss of peripheral heterochromatin in nuclei of HGPS patients' cells expressing the most common LA mutation (LAΔ50). Using a female LAΔ50 patient's cells, we have gained insights into the mechanisms involved in the loss of heterochromatin using the inactive X (Xi) chromosome as a reporter for peripheral heterochromatin. Xi is readily visualized by immunofluorescence using an antibody to the trimethylated K27 on histone H3 (H3K27m3). In mid-passage HGPS cells, H3K27m3 is lost as determined by immunoblotting and there is an apparent loss of Xi by immunofluorescence prior to nuclear envelope lobulation. However, fluorescence *in situ* hybridization (FISH) shows that Xist RNA is retained in these HGPS cells for over 20 passages when nuclei became highly convoluted and LAΔ50 accumulates to high levels. However, in late passage cells the Xist RNA appears to unravel. In contrast, the Xi in cells from the normal female sibling of this patient appears normal for up to 20 passages. To more specifically test whether the accumulation of LAΔ50 causes the loss of H3K27m3, female kidney (HEK293) cells were transfected with pEGFP-LAΔ50 cDNA. Within 12-24 h the Xi in these cells could not be detected with anti-H3K27m3. Controls transfected with pEGFP-LA cDNA showed no loss of Xi staining. In both cases, Xist RNA remained associated with the Xi. There is also a complete loss of peripheral heterochromatin in a stable HeLa cell line expressing GFP-LAΔ50. RTPCR analyses showed that the Ezh2 mRNA which encodes the transmethylase responsible for H3K27 methylation is greatly reduced in mid-late passage HGPS cells. Supported by NIA, PRF and the Ellison Foundation.

## Intersection of Signaling & Trafficking: Small GTPases (1496-1501)

1496

**Disruption of Actin Cytoskeleton Leads to tER-Golgi Splitting in *Drosophila* S2 Cells**

V. Kondylis, B. H. A. Herpers, C. Rabouille; Cell Biology, Cell Microscopy Centre, University Medical Centre Utrecht, Utrecht, The Netherlands

In addition to its central position in intracellular membrane traffic, the Golgi apparatus has been suggested to act as platform for signaling events. Accordingly, there is accumulating evidence showing that several small GTPases of the Ras superfamily and their binding proteins localize on the Golgi membranes, where they could initiate signaling cascades but also maintain/alter the Golgi architecture. The latter seems to be confered by regulating locally the cytoskeleton organization. In *Drosophila* S2 cells, the Golgi apparatus is organized in ~20 individual Golgi stacks closely associated with tER sites (tER-Golgi units). Whereas microtubule depolymerisation does not affect their organization, interference with F-actin dynamics using Cytochalasin D or Latrunculin B results in an approximately 2-fold increase in the number of both the Golgi stacks and tER sites. This has been assessed by immunofluorescence localisation of tethering and Golgi resident proteins, COPII subunits, and cargo receptors, while electron microscopy has revealed that Golgi stacked morphology in these cells remains largely intact. Kinetically, the effect of F-actin depolymerisation on tER-Golgi units takes place within 15-30 min after drug treatment, without significantly affecting anterograde transport to the plasma membrane. The tER-Golgi duplication is protein synthesis and ER-Golgi transport independent, suggesting a splitting rather than a *de novo* mechanism for the formation of the additional organelles. The molecular mechanism involved in tER-Golgi splitting, through F-actin local rearrangements, is currently under investigation. Preliminary results suggest that cdc42- or Rock kinase-mediated changes in F-actin dynamics are not responsible for the effect. Finally, to address the biological significance of this phenomenon, the behaviour of the tER sites and Golgi apparatus during cell cycle is being analyzed.

1497

**Arhgap21, a Novel Rhogap Protein Acting at the Crossroads between Arf1 and Cdc42 Pathways**

P. Chavrier,[1] T. Dubois,[1] N. Ekhatibe,[1] J. Menetrey,[1] M. Perderiset,[1] A. Houdusse,[1] O. Paleotti,[2] M. Franco,[2] A. A. Mironov,[3] M. De Matteis[3]; [1]Cell Biology, Institut Curie, Paris, France, [2]Cnrs umr6097, IPMC, Valbonne, France, [3]Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

ADP-ribosylation factors (ARFs) are a family of GTP-binding proteins involved in the control of membrane trafficking. ARF1 acts as a master regulator of Golgi complex organization and function through the recruitment of various effectors including coat proteins and lipid modifying enzymes. Members of the Rho family, especially Cdc42, are also known to control Golgi structure through the regulation of Arp2/3 complex/F-actin dynamics on Golgi membranes. We identified ARHGAP21 by yeast two-hybrid as a downstream partner of GTP-bound ARF1. Immuno-localization analysis revealed that ARHGAP21 is predominantly associated with the Golgi complex in an ARF1-dependent manner. ARHGAP21 comprises a RhoGAP domain, which preferentially stimulates Cdc42-mediated GTP hydrolysis in vitro. In addition, ARHGAP21 harbors an amino-terminal PDZ domain and a PH domain. The PH domain, although it does not interact with lipids, is required for interaction with GTP-bound ARF1 and Golgi association. The crystal structure of the complex between GTP-ARF1 and the ARF-binding domain of ARHGAP10 revealed that the interface comprises the PH domain and a carboxy-terminal flanking region. The function of ARHGAP21 on the Golgi complex has been assessed by knocking-down the protein by treatment with short interfering RNAs. Loss of ARHGAP21 was shown to induce fragmentation of the Golgi complex and the accumulation of Arp2/3 complex-containing structures in the cytoplasm. In addition, constitutive activation of ARF1 triggered the formation of F-actin and Arp2/3 complex-based structures, which required Cdc42 activity and was inhibited by expression of the RhoGAP domain. We will also provide evidence that a novel RhoGAP protein closely related to ARHGAP21 is able to bind activated ARF proteins. All together, our results establish a role for ARHGAP21-related proteins in the control of membrane trafficking

downstream of ARF proteins through the regulation Cdc42-mediated actin cytoskeleton reorganization.

1498

**Yeast Oxysterol-Binding Protein Homologues Affect CDC42- and RHO1-Mediated Cell Polarization**

K. G. Kozminski,[1] G. Alfaro,[2] C. T. Beh[2]; [1]Depts. Biology and Cell Biology, University of Virginia, Charlottesville, VA, [2]Dept. Molecular Biology & Biochemistry, Simon Fraser University, Burnaby, BC, Canada

Polarized cell growth requires the establishment of an axis of growth (i.e., cell polarity), which is generally followed by targeted secretion to the cell cortex along this established axis. Disrupting the ordered progression of these processes causes a failure in polarized growth, leading to cells with aberrant cell morphology and impaired function. How polarity establishment and secretion are choreographed is not fully understood, though it is known that Rho and Rab GTPase-mediated signal transduction is required for these events. Superimposed on this regulation are the functions of specific lipids and their cognate binding proteins. Using *S. cerevisiae* to screen for genes that promote polarity establishment by interactions with *CDC42*, which encodes a Rho-family GTPase, we identified *KES1/OSH4*, a yeast homologue of the canonical human oxysterol-binding protein (OSBP). We found that several other yeast *OSH* genes (OSBP homologues) had comparable genetic interactions with *CDC42*, implicating the *OSH* gene family in the regulation of *CDC42*-dependent polarity establishment. We also showed that the entire *OSH* gene family (*OSH1 - OSH7*) is necessary for the maintenance of cell polarity and for the proper localization of proteins involved in polarized growth (i.e., bud formation) such as septins and the Rab GTPase Sec4p. However, many proteins involved in the polarized organization of the actin cytoskeleton localized at the bud site independently of *OSH* gene function. Our data showed that although *OSH* genes promote *CDC42*-dependent polarity establishment, they are antagonistic to *RHO1*-dependent secretion, working in opposition to Sec4p GTPase Activating Proteins (Msb3p and Msb4p). Based on our data, we propose a model in which *OSH* gene function licenses polarized secretion only after cell polarity is established.

1499

**Reversible Intracellular Translocation of KRas in Hippocampal Neurons Regulated by Ca2+/Calmodulin**

M. Fivaz, T. Meyer; Molecular Pharmacology, Stanford University, CA, Stanford, CA

The Ras/MAPK pathway regulates synaptic plasticity and cell survival in neurons of the central nervous system. Here, we show that KRas but not HRas rapidly translocates from the plasma membrane to the Golgi complex and early/recycling endosomes in response to neuronal activity. Translocation is reversible and mediated by the polybasic-prenyl membrane targeting motif of KRas. We provide evidence that KRas translocation occurs through sequestration of the polybasic-prenyl motif by $Ca^{2+}$/Calmodulin ($Ca^{2+}$/CaM) and subsequent release of KRas from the plasma membrane, in a process reminiscent of GDP dissociation inhibitor (GDI)-mediated membrane recycling of Rab and Rho GTPases. KRas translocation was accompanied by partial intracellular redistribution of its activity. We conclude that the polybasic-prenyl motif acts as a $Ca^{2+}$/CaM-regulated molecular switch that controls plasma membrane concentration of KRas and redistributes its activity to internal sites. Our data thus define a novel signaling mechanism that differentially regulates KRas and HRas localization and activity in neurons.

1500

**The DOCK180/Elmo Complex Couples Arf6 Activation to the Downstream Activation of Rac1**

L. C. Santy,[1] K. Ravichandran,[2] J. E. Casanova[1]; [1]Department of Cell Biology, University of Virginia, Charlottesville, VA, [2]Department of Microbiology, University of Virginia, Charlottesville, VA

Cell motility requires extensions of the plasma membrane driven by reorganization of the actin cytoskeleton. Small GTPases, particularly members of the Rho-family, are key regulators of this process. A second class of GTPases, the ADP-ribosylation factors (ARFs) have also been implicated in the regulation of the actin cytoskeleton and motility . ARF6 is intimately involved in the regulation of Rac activity, however the mechanisms by which ARF activation leads to activation of Rac remain poorly understood. We have previously shown that expression of the ARF-specific guanine nucleotide exchange factor ARNO in MDCK cells induces robust activation of Rac, the formation of large lamellipodia and the onset of motility. We report here that this Arf6-induced activation of Rac is mediated by a bipartite Rac GEF, the Dock180/Elmo complex. Both DOCK180 and Elmo colocalize extensively with ARNO in migrating MDCK cells. Importantly, both a catalytically inactive Dock180 mutant and an Elmo mutant that fails to couple to Dock180 block ARNO-induced Rac activation and motility. In contrast, a similar mutant of the Rac GEF Î²-PIX fails to inhibit Rac activation or motility in this context. Together, these data suggest that ARNO and ARF6 coordinate with the Dock180/Elmo complex to promote Rac activation at the leading edge of migrating cells.

1501

**NGF Signaling in Neurite Outgrowth via Regulation of Rab5**

J. Liu,[1] Z. Liang,[1] D. Lamb,[1] M. Chou,[2] Y. Liu,[3] G. Li[1]; [1]Biochemistry and Molecular Biology, University of Oklahoma HSC, Oklahoma City, OK, [2]Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA, [3]Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA

NGF induces neurite outgrowth in the differentiation of PC12 cells. This process involves NGF binding to its receptor TrkA and endocytosis of the NGF-TrkA complex into signaling endosomes where the differentiation signal is propagated and sustained. In this study, we find that this process requires inactivation of Rab5 to shut down early endosome fusion, allowing TrkA-containing endocytic vesicles to mature/specialize into signaling endosomes. NGF treatment led to decreases in the cellular level of active GTP-bound Rab5, as shown by pull-down assays with the Rab5 effector Rabaptin5. Importantly, we identified a novel Rab5 GAP (RabGAP5) that was associated with TrkA in co-immunoprecipitation assays and was responsible for the decrease of Rab5-GTP level in the cell. Expression of a GAP-dead RabGAP5 mutant blocked neurite outgrowth in PC12 cells. Furthermore, expression of a dominant negative Rab5 mutant (Rab5:S34N) enhanced NGF-induced neurite outgrowth, while expression of a dominant positive Rab5 mutant (Rab5:Q79L) to force early endosome fusion abolished this differentiation process. We further confirmed these results by establishing stable PC12 cell lines expressing Rab5:S34N. The neurite outgrowth in these cells occurred much earlier than in parental PC12 cells. Taken together, the results suggest that NGF down-regulates Rab5 via RabGAP5 to facilitate neurite outgrowth and cell differentiation. This process is in contrast to EGF-induced cell growth where EGF signals through RIN1, a Rab5 GEF, to activate Rab5.

## Mitosis & Meiosis (1502-1507)

1502

### Studying Spindle Assembly and Chromosome Condensation in *Xenopus* Egg Extracts

R. Heald, R. Deehan, B. S. Freedman, E. Hannak, T. J. Maresca, M. D. Blower, K. S. Brown, T. C. Grammer, R. M. Harland; Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Accurate segregation of the genetic material during mitosis and meiosis requires a dramatic restructuring of both the chromosomes and the microtubule cytoskeleton to form a dynamic mitotic spindle. We have utilized functional reconstitution approaches in *Xenopus laevis* egg extracts to identify mechanisms underlying the complex events of chromosome condensation and spindle assembly, and to elucidate roles of the individual factors involved. Current projects investigate the contribution of linker histone H1 and the cohesin complex to higher order mitotic chromosome architecture, and explore the role of microtubule associated proteins such as the *Xenopus* CLASP homologue, Xorbit, in coupling microtubule plus end dynamics to kinetochore movement and chromatin-driven microtubule polymerization. To expand our repertoire of in vitro approaches, we have developed the use of egg extracts from a related frog, *Xenopus tropicalis*, a diploid species amenable to genetic approaches and for which genomic sequence data are available to facilitate rapid proteomic analyses. Interestingly, we have found that metaphase spindles formed in *X. tropicalis* extracts are approximately 30% smaller than *X. laevis* spindles, although the same chromosome source (*X. laevis* sperm nuclei) was used in each assembly reaction. This observation suggests that cytoplasmic activities contained within each extract define spindle length. In support of this hypothesis, mixing experiments with the two types of extract yielded intermediate spindle sizes with a linear relationship between the percentage of each extract in a reaction and the final spindle length. Thus, in addition to providing a more molecularly tractable system, experiments with *X. tropicalis* egg extracts may provide novel insight into the regulation of spindle morphogenesis.

1503

### Centromere Pairing and Chromosome Segregation at Meiosis I

M. Stewart, D. Dawson; Department of Molecular Biology/Microbiology, Tufts University School of Medicine, Boston, MA

Our experiments are intended to explore the mechanisms used to partition chromosomes at meiosis I. One of the key differences between meiotic and mitotic chromosome segregation lies in the behavior of homologous chromosomes at meiosis I. Homologous pairs experience recombination (exchanges) prior to metaphase I. These exchanges between the are important for linking the chromosomes, which allows them to attach to the spindle in a bipolar orientation. Exchanges (along with sister chromatid cohesion distal to the exchnage) hold homologs together until the signal for anaphase is received. Mutations that eliminate meiotic recombination result in high levels of meiosis I non-disjunction, and consequently, sterility or reduced fertility. However, in many eukaryotes, single chromosome pairs that fail to experience crossovers do not segregate randomly. This indicates that mechanisms beyond exchange can facilitate meiosis I segregation. Our experiments have focused on examining the mechanisms used to partition non-exchange chromosomes in budding yeast. We expect that these mechanisms probably work in conjunction with crossovers to insure proper segregation of all chromosome pairs. Our experiments revealed that the centromeres of non-exchange chromosomes are actively paired in prophase of meiosis I. This centromere pairing appears to hold non-exchange chromosomes together and orient the kinetochores towards opposite poles. We propose that pairing of non-exchange chromosomes is a default process that occurs after exchange homologs have synapsed. Preliminary results show that centromeric cohesion, the synaptonemal complex, and monopolin all play roles in the centromere pairing of non-exchange chromosomes, and may help play a similar role for exchange chromosomes, helping to orient their centromeres so that they attach properly to the meiosis I spindle.

1504

### A Conserved Checkpoint Monitors Meiotic Chromosome Synapsis in *C elegans*

N. Bhalla, A. F. Dernburg; E.O. Lawrence Berkeley National Lab, University of CA, Berkeley, Berkeley, CA

Meiosis generates haploid gametes from a diploid cell by coupling a single round of replication with two successive rounds of chromosome segregation. During meiotic prophase, the pairing and synapsis of homologous chromosomes and interhomolog recombination are critical events that ensure the proper disjunction of chromosomes. We are studying the mechanisms that monitor these events and contribute to their accuracy. Previous work in *C. elegans* and other organisms has shown that DNA damage that is not repaired during meiotic recombination can target meiotic nuclei for apoptosis. We have now found evidence for a second checkpoint that leads to apoptosis in the germline: errors in synapsis, even in the absence of DNA damage, can act as a direct trigger for cell death. When we analyze mutants where one pair of chromosomes fail to synapse or all chromosomes exhibit asynapsis, we observe elevated levels of germline apoptosis. This activation of apoptosis in response to unsynapsed chromosomes is independent of double-strand break (DSB) formation and the germline DNA damage checkpoint, distinguishing it from the pachytene checkpoint in budding yeast. However, the *C. elegans* homolog of the yeast checkpoint component *PCH2*, is required for synapsis checkpoint activation, suggesting that the molecular mechanism that detects unsynapsed homologous chromosomes is conserved. Our investigations have also revealed that Pairing Centers (PCs), *cis*-acting chromosomal sites that promote homolog synapsis, are required for this meiotic synapsis checkpoint. The essential role of these *cis*-acting sites in both initiating synapsis and detecting defects in this process is evocative of the involvement of centromeres in both microtubule attachment and the spindle assembly checkpoint. We are interested in identifying additional components of this checkpoint to gain a better understanding of how defects in synapsis are monitored and result in apoptosis.

1505

### Condensin is Essential to Prevent Premature Chromosome Decondensation in Anaphase

P. Vagnarelli, D. F. Hudson, W. Earnshaw; Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom

The mechanisms underlying the establishment of mitotic chromosome condensation and its maintenance until the end of mitosis remain two of the classic unanswered questions of cell biology. It has recently been proposed that two protein complexes called condensin I and II are important for this condensation, and are essential for topoisomerase II to separate sister chromatids during anaphase. We have used a gene targeting analysis to determine the essential function of condensin in chicken DT40 cells. Our results differ significantly from those predicted by present models of condensin function. Condensin is not essential for mitotic chromosomes to adopt an orderly higher-order structure, for normal cohesin dynamics or

for sister chromatid separation during anaphase. Neither is it essential for topo II function at centromeres or on the chromosome arms. Instead, condensin is essential for vertebrate chromosomes to remain condensed throughout anaphase. In condensin-depleted cells chromatids abruptly decondense while still moving during anaphase B. However, chromatids can remain condensed and segregate normally provided that CDK levels are kept high during mitotic exit. Our results identify a new activity, RCA (regulator of condensation in anaphase), that cooperates with condensin to maintain mitotic chromosome condensation in anaphase.

1506

### A Potential Direct Role for the Chromosomal Passenger Complex in Centromere-Microtubule Attachment in *S cerevisiae*

S. Sandall,[1,2] F. F. Severin,[3] A. A. Hyman,[3] A. B. Desai[1,2]; [1]University of California, San Diego, La Jolla, CA, [2]Ludwig Institute for Cancer Research, La Jolla, CA, [3]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

Kinetochores function as the primary chromosomal attachment sites for spindle microtubules. The 125 bp sequence-specific *S. cerevisiae* centromere has proven invaluable for characterizing kinetochore composition and function. Over 60 budding yeast kinetochore proteins have been identified, many of which are conserved in higher organisms. Nevertheless, how these proteins form the interface with spindle microtubules is poorly understood. We have utilized an in vitro approach to identify proteins required for the interaction of budding yeast centromeric (CEN) DNA with microtubules. Extracts of wild-type or mutant yeast strains are incubated with fluorescent beads coated with CEN DNA. The bead/extract mix is then passed over microtubules adsorbed to a flow cell surface and microtubule-bound beads are counted using automated image analysis. The attachments are not formed if beads are coated with mutant CEN DNA that is non-functional in vivo. Using this assay in conjunction with activity-based biochemistry, mass spectrometry of a partially purified fraction, and analysis of candidate mutants, we have identified Sli15 and Bir1, two subunits of the chromosomal passenger complex homologous to INCENP and Survivin in higher eukaryotes, as being necessary for CEN DNA-microtubule attachment *in vitro*. From extensive analysis of mutants, these are the only proteins other than subunits of the CEN DNA-binding CBF3 complex that are required for the activity detected in the assay. Previous work has shown that Ipl1, the Aurora B like kinase subunit of the yeast passenger complex, regulates CEN DNA-microtubule attachment in vitro but does not suggest a direct involvement for the chromosomal passenger complex in the attachment. Our results point to a previously unsuspected direct role for this conserved complex in kinetochore-spindle interactions in budding yeast and also suggest potentially distinct functions for different subunits of the complex.

1507

### Cell Cycle Dependent Chromatin Proteomics: Identification and Characterisation of Novel Xenopus and Human Kinetochore Proteins

I. M. Porter,[1] G. A. Khoudoli,[1] J. S. Andersen,[2] J. J. Blow,[1] J. R. Swedlow[1]; [1]Division of Gene Regulation & Expression, University of Dundee, Dundee, United Kingdom, [2]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Many protein factors involved in chromosome segregation are known, but many more factors likely remain to be identified and studied. We have therefore examined the proteome of chromatin and chromosomes at specific stages of the cell cycle. Interphase chromatin and mitotic chromosomes were generated in vitro by incubating sperm nuclei in Xenopus egg cytoplasmic extracts. After isolation of chromatin and chromosomes, associated proteins were eluted and soluble protein fractions were subjected to LC/LC-MS/MS or 2D gel electrophoretic analysis. LC/LC-MS/MS analysis identified over 500 chromatin bound proteins, with approximately 150 specific to mitotic chromosomes. We have used an RNAi-based live HeLa cell secondary screen to characterise the properties of a number of proteins identified in the proteomic analysis that were either entirely novel or poorly characterised. An shRNA-expressing vector (pU6YH) that co-expresses a specific shRNA and H2B-YFP was used to simultaneously identify those cells producing shRNA and monitor any mitotic phenotype by 3D time-lapse fluorescence microscopy. Of 12 uncharacterised proteins, 4 gave strong mitotic phenotypes. One protein has striking sequence similarities to S. cerevisiae Dam1p, a critical kinetochore protein required for proper chromosome attachment and biorientation in yeast. When expressed as a GFP fusion, the human Dam1p homologue localises to spindle poles in G2 and M phase cells and outer kinetochores from prometaphase to anaphase. siRNA against hDam1 in HeLa cells causes a strong biorientation defect, increases the amount of tubulin polymer detected in fixed cells by 4.5x and the amount of chTOG associated with mitotic spindles by 2.5x. We generated a polyclonal antibody that recognises both the human and Xenopus Dam1 proteins, and immunoprecipitates XDam1, XMAP215, and CENP-E from Xenopus CSF extracts. Studies to examine microtubule dynamic after Dam1 depletion and knockdown are underway.

## Organelle Dynamics (1508-1513)

1508

### Physiological Functions of Mitochondrial Fusion in Mice

H. Chen, D. C. Chan; Division of Biology, California Institute of Technology, Pasadena, CA

Mitochondria are dynamic organelles that constantly fuse and divide. These processes control the overall morphology of the mitochondrial population within cells, and likely have important roles in regulation of mitochondrial function. Recent studies have implicated mitochondrial fission in the progression of programmed cell death. In addition, two human neuropathies (Charcot-Marie-Tooth subtype 2A and dominant autosomal optic atrophy) are caused by mutations in mitofusin 2 (Mfn2) and OPA1, proteins essential for mitochondrial fusion. To understand the physiological role of mitochondrial fusion in mammals, we have generated mice carrying null or conditional alleles of Mfn1 and Mfn2. Analysis of mutant mice and cells indicate that mitochondrial fusion controls not only mitochondrial morphology but also mitochondrial heterogeneity and function. The role of mitofusins in membrane fusion will also be discussed.

1509

### Interplay of SNAREs and a dynamin-like GTPase in vacuole fission and fusion

A. Mayer, T. Baars, C. Peters; Department of Biochemistry, University of Lausanne, Epalinges, Switzerland

Membrane traffic consists of cycles of membrane fission and fusion. Fission and fusion must be balanced to guarantee membrane homeostasis - and they are intrinsically antagonistic reactions. Activated fusion machinery might immediately reverse membrane fission of a nascent vesicle, and vice versa, resulting in futile cycles of fission and fusion. This poses the problem if and how these antagonistic activities are coordinated to

guarantee efficient net fusion or fission when needed. Yeast vacuoles undergo regulated cycles of membrane fission and fusion which are linked to the cell cycle. Vacuole fusion can be reconstituted in a cell free system allowing detailed analysis of its mechanisms. It shares many key features with fusion reactions in higher eukaryotic organisms, making it an attractive model. Recently, we have identified a dynamin-like GTPase, Vps1p, as a factor required for vacuole fission. At the same time, however, Vps1p is required for vacuole fusion and interacts functionally and physically with parts of the vacuolar fusion machinery, SNAREs and their activating ATPase Sed18p/NSF. The roles of Vps1 and of SNARE proteins in membrane fusion and fission at the vacuole will be discussed.

1510

**VTC Assembly by Homotypic COPII Vesicle Fusion Involves an Unanticipated Function for Uncomplexed SNAREs in Tether Recruitment**

K. Mullen,[1] D. Xu,[2] J. C. Hay[1]; [1]Division of Biological Sciences, University of Montana, Missoula, MT, [2]EMBL, Heidelburg, Germany

What is the first membrane fusion step in the secretory pathway? In mammals, COPII-coated transport vesicles deliver secretory cargo to VTCs that ferry cargo from ER exit sites to the Golgi. However, the precise origin of VTCs and the membrane fusion step(s) involved have remained experimentally intractable. Here we document in vitro direct tethering and SNARE-dependent homotypic fusion of ER-derived COPII transport vesicles to form larger cargo containers. The assembly did not require detectable Golgi membranes, pre-existing VTCs, or COPI function. Thus, COPII vesicles appear to contain all of the machinery to initiate VTC biogenesis via homotypic fusion. Antibodies that block SNARE complex assembly inhibited homotypic fusion without severely affecting the upstream tethering event, consistent with the well-characterized role of SNARE complexes in fusion. However, inhibition of SNARE complex disassembly by NSF caused a significant decrease in homotypic COPII vesicle tethering as well as fusion, implying an unanticipated role for uncomplexed SNAREs in tethering. Examination of fusion intermediates revealed that COPII vesicles lacking uncomplexed SNAREs failed to recruit the tether GM130 and other regulatory molecules from the cytosol. Our results indicate that uncomplexed SNAREs provide a "signal" that constrains tether assembly to sites containing fusogenic SNAREs. Although it has often been stated that tethering proceeds upstream of and independently of SNAREs, our results indicate that information passes in both directions between tethers and SNAREs. Elucidation of the pathway linking uncomplexed SNAREs to tether recruitment will be described.

1511

**Examining Golgi Maturation in Living Yeast**

E. Losev, C. A. Reinke, J. Jellen, D. E. Strongin, B. S. Glick; MGCB, University of Chicago, Chicago, IL

The Golgi apparatus is made up of biochemically distinct early (*cis*, *medial*) and late (*trans*, TGN) cisternae. The nature of these cisternae has been a subject of debate. The stable compartments model predicts that each Golgi cisterna is a long-lived compartment that retains a characteristic set of Golgi resident proteins. Conversely, the cisternal maturation model predicts that each cisterna matures from early to late by acquiring and then losing specific Golgi resident proteins. We performed a direct test of these two models using 4D video microscopy. The early Golgi was labeled by tagging Vrg4p with GFP, and the late Golgi was labeled by tagging Sec7p with monomeric DsRed. We observed that individual early Golgi cisternae matured into late Golgi cisternae. Alpha-factor pulse-chase experiments, were used to estimate the time required for a secretory protein to transit the Golgi. The kinetics of intra-Golgi transport as measured by pulse-chase match the kinetics of Golgi maturation as measured by video microscopy, confirming that the cisternal maturation model can account for anterograde traffic through the Golgi.

1512

**Centriole Assembly in C elegans**

L. Pelletier,[1] E. T. O'Toole,[2] A. Schwager,[1] A. A. Hyman,[1] T. Mueller-Reichert[1]; [1]Max Planck Institute CBG, Dresden, Germany, [2]The Boulder Laboratory for 3D Electron Microscopy of Cells, Department of Molecular, Cellular, and Developmental Biology, Boulder, CO

Genome-wide RNAi screens and classic genetic analyses in the nematode C. elegans have led to the identification of proteins required for centriole duplication in the early embryo. These proteins include SPD-2, ZYG-1, SAS-4, SAS-5 and SAS-6. Using EM tomography of high-pressure frozen single embryos in combination with GFP-based centriole assembly assays and RNAi, we analyzed the centriole duplication cycle during the first cell division of the C. elegans embryo. Our results show that in wild-type embryos centriole duplication is initiated at the onset of centrosome separation. At this stage a daughter centriole precursor consisting of an inner cylindrical tube of reduced length lacking singlet microtubules can be seen in close proximity to both mother centrioles. During pronuclear migration the inner tube elongates reaching its full length prior to pronuclear rotation. During rotation, the 9 singlet microtubules begin assembling around the inner tube, a process completed prior to metaphase where a fully duplicated centriole pair can be observed at each pole of the mitotic spindle. Using GFP-based mating assays we find that centriole components are recruited to centrioles in two temporarily and spatially distinct steps. Shortly after fertilization, during female meiosis, SPD-2 and ZYG-1 are recruited to the mother centrioles. We further show that SPD-2 is required to localize ZYG-1 to the mother centrioles suggesting that SPD-2 acts upstream of ZYG-1 in this pre-duplication event. Shortly after meiosis, at the time of pronuclear appearance, SAS-4, SAS-5 and SAS-6 become recruited in close proximity of the mother centriole, to what is likely to be the forming daughter centriole. We are currently in the process of reconstituting centriole assembly intermediates by EM tomography upon RNAi of the known centriole components.

1513

**The Cilium/flagellum: A Secretory Organelle?**

K. Huang,[1] D. R. Diener,[1] R. Karki,[1] L. B. Pedersen,[1] S. Geimer,[2] J. L. Rosenbaum[1]; [1]MCDB Department, Yale University, New Haven, CT, [2]Biology/Electron Microscopy, University of Bayreuth, Bayreuth, Germany

The membranes of cilia and flagella are formed from Golgi-derived vesicles targeted for exocytosis at a location ajdacent to the ciliary/flagellar basal bodies. There is no known mechanism for the reclamation of ciliary membranes, so excess ciliary membrane must be discarded. We believe that the cilium/flagellum is not only a sensory and motile organelle, but that it probably also functions as a secretory organelle, by exocytosis of membrane vesicles from its tip. This “secretion,” by pinching off of vesicles, probably balances the addition of membrane at the base of the cilium. We have isolated membrane vesicles from Chlamydomonas medium, and compared them to highly purified membrane vesicles isolated from Chlamydomonas flagella. The vesicles that are “secreted” into the medium contain an enrichment of a subset of the polypeptides found in

membranes obtained from isolated flagella, and include certain intraflagellar transport (IFT) polypeptides, IFT motors, tubulin, 14-3-3 protein, PKD-2 (polycystin-2) and other polypeptides. They do not contain certain axonemal proteins that are being delivered to the flagellar tip by IFT, *eg* radial spoke proteins. By use of fluorescent Con A, which binds to flagellar membrane glycoproteins, dyes that stain membrane lipids, and mutants defective in IFT, we have been able to show that, *in vivo,* membrane lipids and proteins are actively moved to the tip of the flagellum by IFT, and can be followed into the medium. Correlated EM thin sections support this vesicle secretion action at the tip. We presume that this "secretory" activity occurs in almost all cilia, including the solitary sensory primary cilia of vertebrate cells, cilia of the respiratory and reproductive tracts, and the sensory cilia of C. elegans. Supported by NIH grants GM14642.

## Protein Misfolding & Disease (1514-1519)

1514

### Mechanism of alpha -Synuclein toxicity: Inhibition of Endoplasmic Reticulum-Golgi Trafficking

A. A. Cooper,[1] A. Gitler,[2] C. M. Haynes,[1] K. J. Hill,[1] A. Cashikar,[2] S. Lindquist[2]; [1]Division of Cell Biology and Biophysics, University of Missouri-Kansas City, Kansas City, MO, [2]Whitehead Institute for Biomedical Research, MIT, Cambridge, MA

Parkinson's disease (PD) is one of the most common human neurodegenerative disorders, affecting approximately 2% of people after age 65 and about 5% after age 85. Accruing evidence points to a role for the protein alpha-synuclein (αSyn) in the pathogenesis of PD but little is known about its regular cellular function or its contribution to the disease. In *Saccharomyces cerevisiae*, increased αSyn expression is cytotoxic and the underlying cause(s) were examined in an effort to explain the etiology of PD. Here we show that lethality is due to a complete block in endoplasmic reticulum (ER)-Golgi vesicular trafficking. Such a block causes ER stress, a condition also associated with PD, as evidenced by activation of the unfolded protein response. Finally, a direct cause/effect relationship between impaired ER-Golgi trafficking and cytotoxicity was demonstrated by the suppression of αSyn associated cellular defects through over-expression of a subset of ER-Golgi trafficking components. Our finding that the primary defect in cells accumulating αSyn is impaired vesicular trafficking likely explains the particular vulnerability of dopamine producing neurons in PD and will likely open novel avenues for therapeutic intervention.

1515

### Native Amyloid in the Secretory Lysosome Pathway - Lessons for Disease

D. M. Fowler,[1] A. V. Koulov,[2] C. Alory-Jost,[2] M. S. Marks,[3] J. W. Kelly,[1] W. E. Balch[2]; [1]Department of Chemistry and the Skaggs Institute of Chemical Biology, The Scripps Research Institute, La Jolla, CA, [2]Department of Cell Biology and the Institute for Childhood and Neglected Diseases, The Scripps Research Institute, La Jolla, CA, [3]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA

Proteins are biopolymers that typically adopt a well-defined three-dimensional structure, but can misfold and form aggregates with a specific cross beta-sheet fold called amyloid. This multistep process is linked to a number of diseases, including many resulting in neurodegeneration, whose unifying pathological feature is the presence of protein aggregates with the amyloid structure. Functional, non-pathogenic amyloid has not previously been detected in higher organisms and was unexpected due to the toxicity associated with amyloid formation. We have discovered an abundant, native, intracellular, mammalian amyloid structure that functions in melanosome biogenesis, challenging the current view that intracellular amyloid in mammals is always pathologic. Melanosomes are organelles that contain fibers composed of the protein Pmel. They function to template the synthesis of the tyrosine-based polymer melanin, which protects mammals from UV damage. We have shown that the Pmel fibers in isolated mammalian melanosomes have an amyloid structure based on the binding of dyes that fluoresce upon interacting with a cross beta-sheet structure, and on our ability to reconstitute Pmel amyloid formation *in vitro*. Interestingly, the rapidity of recombinant Pmel fibrilization is unprecedented, consistent with a process optimized by evolution for function and to avoid the toxicity of pathological amyloidogenesis. Our results suggest that variations in the structure and stability of amyloid and the context (environment) in which it is generated by the cell distinguishes function from pathology. The utilization of the amyloid fold for a major cellular activity in mammals demonstrates that sequence and folding pathway evolution can harness this ancient structure for physiological purposes.

1516

### A Molecular Mediator of Neurodegeneration in the Polyglutamine Disease SCA1

H. Orr; Institute of Human Genetics, University of Minnesota, Minneapolis, MN

Spinocerebellar ataxia type 1 (SCA1) is a member of a group of inherited neurodegenerative diseases caused by a CAG trinucleotide expansion that results in the expansion of a polyglutamine tract at the protein level. Here we present data that demonstrate an interaction between wild type Atx-1 (Atx-1) and the retinoid-related orphan nuclear receptor alpha (RORa) and Tip60 a coactivator of RORa.. RORa is a transcription factor highly expressed in Purkinje cells. Loss of RORa is the basis of the cerebellar atrophy seen in staggerer mice. Expansion of the polyglutamine tract in Atx-1 that induces a conformational change and a corresponding decrease in its interaction with RORa and increase in its interaction with Tip60. In SCA1 mice expression of mutant Atx1 resulted in a depletion of RORa and a reduction in expression of genes controlled by the RORa complex. Moreover, loss of one allele of RORa enhanced the pathogenicity of mutant Atx-1 in vivo. These results implicate the loss of RORa as one molecular mechanism of SCA1 pathogenesis that addresses several key aspects of SCA1.

1517

### Inducible Tauopathy in Neuronal Cell Models Shows that the Aggregation of Microtubule-Associated Protein Tau is Toxic to Cells

I. Khlistunova, J. Biernat, Y. Wang, M. Pickhardt, Z. Gazova, E. M. Mandelkow, M. Von Bergen, E. Mandelkow; Structural Molecular Biology, Max-Planck-Institute, Hamburg, Germany

We have generated several models of tau pathology in cells in order to study tau's mechanisms of neurodegeneration in Alzheimer's and other brain diseases. N2a neuroblastoma cell lines were created that inducibly express different variants of tau when exposed to doxycyclin (tet-on system). Three constructs were chosen, based on the repeat domain of full-length tau: The 4R wild-type repeat domain (K18), the 4R repeat domain with the deletion mutation deltaK280 known from frontotemporal dementia and highly prone to spontaneous aggregation, and the 4R repeat domain with

deltaK280 and two further proline point mutations that strongly inhibit aggregation. The comparison of these wild-type, pro-aggregation and anti-aggregation mutants shows: (1) Tau aggregation into paired helical filaments is toxic to cells, (2) the degree of aggregation and toxicity strongly depends on tau's propensity for beta-structure, (3) soluble tau mutants that cannot aggregate are also not toxic; (4) tau phosphorylation in the repeat domain (at KXGS motifs) precedes aggregation but is not correlated with the degree of aggregation; (5) tau aggregates disappear when the tau expression is silenced, showing that aggregation is reversible; (6) Tau aggregation can be prevented by drugs, and even pre-formed aggregates can be dissolved again by drugs. Thus, the cell models open up new insights into the relationship between tau structure, expression, phosphorylation, aggregation, and toxicity that can be used to test current hypotheses on tauopathy and to develop drugs that prevent the aggregation and degeneration of cells. - Supported by ISOA and DFG.

1518

**FAD-Linked Presenilin1 Mutations Show Trafficking Defects from the Endoplasmic Reticulum**

J. Kim,[1] G. Thinakaran,[2] S. S. Sisodia,[2] R. Schekman[1]; [1]Department of Molecular and Cell Biology, University of California-Berkeley, Berkeley, CA, [2]Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, Chicago, IL

Familial Alzheimer's disease (FAD)-linked presenilin (PS) mutations are scattered throughout the PS structural gene. A similar pattern has been found in the genes of other integral membrane proteins linked to human diseases (Sanders and Myers, 2003). These mutated proteins show misassembly and/or trafficking defects in the endoplasmic reticulum (ER). We hypothesized that FAD-linked PS1 mutations cause misfolding of PS1, resulting in aberrant trafficking of PS1 from the ER. Using a cell free assay to directly monitor the exit of cargo proteins from the ER, we show that some of FAD-linked PS1 variants are inefficiently packaged into transport vesicles. An extreme allele, PS1ΔE9, also causes abnormal packaging of APP *in vitro*, and inhibits glycosylation of nicastrin (Nct) *in vivo*, suggesting retention of the γ-secretase complex in the ER. Trimethylamine N-oxide (TMAO), an agent known to influence protein folding, partially rescues abberant trafficking of PS1 and abnormal glycosylation of Nct. TMAO treatment of Neuro 2a cells expressing PS1ΔE9 reduces the ratio of $A\beta_{42}/A\beta_{40}$. Our results suggest that misfolding and/or retention of PS1 in the ER is linked to the generation of toxic $A\beta_{42}$ peptide.

1519

**ER Quality Control Beyond ERAD & the UPR : Autophagy Takes Out the Aggregated Garbage**

K. B. Kruse,[1] A. Dear,[2] E. R. Kaltenbrun,[1] B. E. Crum,[1] J. L. Brodsky,[3] P. M. George,[2] S. O. Brennan,[2] A. A. McCracken[1]; [1]Biology, University of Nevada, Reno, Reno, NV, [2]Molecular Pathology Lab, Christchurch School of Medicine and Health Sciences, University of Otago, Christchurch, New Zealand, [3]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA

The Z variant of human alpha-1 proteinase inhibitor (A1PiZ) is a substrate for endoplasmic reticulum-associated protein degradation (ERAD) in yeast and mammalian cells. However, we demonstrate here that when A1PiZ is over expressed in yeast, ERAD is saturated and the excess A1PiZ that forms aggregates in the ER is targeted to the vacuole via autophagy. These findings correlate with the pathology of individuals homozygous for A1PiZ. They develop emphysema through a loss-of-function mechanism due to selective removal of the aberrant polypeptide, and a subset of these individuals also experience liver disease through a gain-of-function mechanism when A1PiZ accumulates and aggregates within the ER of their hepatocytes. Interestingly, individuals carrying a mutant variant of the plasma protein fibrinogen present with a similar phenotype, i.e. low plasma levels of fibrinogen, and a subset of individuals who also experience liver disease due to the accumulation of fibrinogen within the ER of their hepatocytes. Because of the similarities between the two disease phenotypes, we hypothesized that the same cellular mechanisms were responsible for the clearance of both A1PiZ and mutant fibrinogen polypeptides. Here we demonstrate that a mutant variant of the fibrinogen gamma chain is degraded through the ERAD pathway, and that when this process is saturated aggregates form within the ER that are cleared by way of autophagy. Together, our findings demonstrate that our yeast system is a valuable model system for the study of autophagy and its link to ER quality control. Further, our findings may have application in the understanding of, and treatment for, individuals with liver disease caused by the accumulation of aggregated polypeptides within the ER of hepatocytes. (Supported by NSF grant MCB-011079.)

## Keith R. Porter Lecture (1520)

1520

**Morphogenesis of a Transport Vesicle**

R. W. Schekman; Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Genetics, biochemistry and morphology have been used to understand the mechanism of protein secretion in eukaryotic cells. Membrane and secretory proteins are sorted in the secretory pathway by cytoplasmic coat proteins that assemble on the surface of a donor membrane, bud transport vesicles, and convey cargo proteins to a target membrane. The COPII coat is responsible for all anterograde traffic of cargo from the endoplasmic reticulum to the Golgi complex. COPII assembly is initiated by the recruitment of the GTP-binding protein, Sar1, to the ER membrane through a transient interaction with a guanine nucleotide exchange factor, Sec12, located on the ER. Sar1-GTP extends an amphipathic N-terminal helix that embeds in the ER bilayer and deforms the membrane into a pleiomorphic tubular carrier. Tethered Sar1 recruits Sec23/24p, which binds potential cargo proteins but not resident ER membrane proteins. Cargo-coat complexes are then clustered by Sec13/31p, which acts as a scaffold to concentrate cargo molecules. The N-terminal helix of Sar1p completes the fission process separating cargo-enriched vesicles from resident proteins that remain behind in the ER. Essentially all cargo proteins may be accommodated in small (70nm) transport vesicles. However some particles and structural proteins must be packaged in larger carriers. Collagen fibers and large lipoproteins, such as chylomicrons, are likely to employ an alternative assembly of COPII for their transport from the ER. Sar1p has been implicated in a rare genetic form of a lipid absorption disease known as Anderson's disease or chylomicron retention disease (CMRD). The disease locus corresponds to one of two human SAR1 genes. SAR1b may regulate the traffic of chylomicrons that are unusually large (~500 nm) in relation to the diameter of a typical COPII vesicle. Sar1a and 1b may differ in their influence on the generation of membrane curvature.

## Oncogenes & Tumor Suppressors (1521-1541)

1521

### The Selectivity in Ras Signaling

C. Cheng,[1] B. Onken,[1] M. Philips,[2] E. Chang[1]; [1]Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, [2]New York University School of Medicine, New York, NY

The Ras G-protein signaling pathways are very complex in humans. There are four Ras proteins. The N-termini of these Ras proteins are over 90% identical in primary sequences, which include the effector-binding domains, and *in vitro* they interact with many effectors with nearly the same efficiency. However, how a given Ras protein selectively activates a particular effector is poorly understood. One leading model suggests that the C-termini of Ras proteins undergo specific post-translational modification, e.g., lipidation, and that such modifications allow Ras to localize and signal from at least two distinct compartments: the plasma membrane and the endomembrane. To unambiguously test this model, we have turned to the fission yeast *Schizosaccharomyces pombe*, which has only one Ras protein, but it is still capable of controlling two highly conserved pathways. Our data show that *S. pombe* Ras selectively activates a MAP kinase pathway to mediate pheromone signaling at the plasma membrane, while it activates Cdc42 at the endomembrane to control cell polarity and mitosis. Ras also appears to act via Cdc42 to transform mammalian cells, and Cdc42 can be found at the endomembrane. Together, these results suggest that Ras in mammalian cells can interact selectively with Cdc42 in the endomembrane. In the current study, we plan to test this idea and to determine if such interaction between Ras and Cdc42 may be important for tumor formation.

1522

### Effects of *Ras* Oncogene on Subcellular Location Patterns in NIH 3T3 Cells

A. T. Hsieh,[1] T. Zhao,[2] T. S. Nowicki,[3] E. Garcia Osuna,[2] R. F. Murphy[4]; [1]Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, [2]Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, [3]Biology, Dickinson College, Carlisle, PA, [4]Biological Sciences and Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA

A systematic approach to protein location analysis was applied to studying the effects of a cancer-promoting gene. This allows us to gain more insight into the mechanism of cancer, specifically in the change of distribution of proteins in cells. A method was developed in our lab to describe subcellular location patterns from fluorescence microscope images numerically, avoiding personal bias and low reproducibility and accuracy that result from visual inspection. These descriptions are based on numerical features, which capture the essence of subcellular location patterns without being sensitive to the extensive variation that occurs in size, shape, and orientation of the cells in microscope images. *Ras*, which is mutated in 30% of human cancers, serves as the model oncogene in this study. NIH 3T3 cell lines with unique proteins tagged with green fluorescent protein (GFP) were transfected with a retroviral vector containing the *Ras* oncogene. Images were collected for the wildtype and *Ras*-containing pairs so the effects of *Ras* oncogene on subcellular location patterns could be examined. A set of numerical features were calculated for each image, and the Friedman and Rafsky (FR) test was performed to determine the change in subcellular location pattern. Of the 20 pairs of cell lines imaged, 14 were found to have statistically significant differences in subcellular distributions between $Ras^+$ and $Ras^-$. These proteins, having distinct properties compared to their wildtype counterparts, are candidates for screening and possible treatment for cancer.

1523

### p53 Functions as a Multidrug Sensitivity Gene in Childhood Neuroblastoma

C. Xue,[1] C. Flemming,[1] K. V. Gurova,[2] R. B. Lock,[1] K. L. Mackenzie,[1] G. M. Marshall,[1] A. V. Gudkov,[2] M. Haber,[1] M. D. Norris[1]; [1]Children's Cancer Institute Australia for Medical Research, Sydney, Australia, [2]Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

The p53 tumor suppressor pathway is the major cancer preventive mechanism in mammals. p53 exerts its tumor suppressor function by controlling genomic stability in cell populations and by suppressing growth of cells with damaged DNA (Gudkov et al, Nat Rev Cancer, 3:111-29, 2003). For pediatric cancers like neuroblastoma, the most common solid tumor of infancy, p53 mutations are rare at diagnosis, but may be acquired following chemotherapy suggesting a potential role in drug resistance. To address this possibility, we generated p53-deficient variants from a panel of p53 wild type tumor cell lines of neuroblastoma and non-neuroblastoma origin by transduction of p53-suppressive constructs encoding either siRNA or a dominant negative p53 mutant. Analysis of these cells indicated that: (i) in contrast to previous reports, wild type p53 was fully functional in all neuroblastoma cell lines tested, as evidenced by its activation and stabilization in response to DNA damage, nuclear transport, transactivation of target genes and control of cell cycle checkpoints; (ii) inactivation of p53 in neuroblastoma cells resulted in the establishment of a broad drug resistant phenotype; (iii) knockdown of mutant p53 did not revert this phenotype suggesting that it is determined by p53 inactivation rather than gain of mutant p53 function; (iv) in contrast to neuroblastoma, p53 suppression had no effect or caused increased drug susceptibility in several other tumor cell types, including renal cell carcinoma. Thus, p53 inactivation is sufficient for the establishment of a multidrug resistant phenotype in neuroblastoma, suggesting that it may be an important prognostic marker of treatment response in this disease. However, p53 suppression may have opposite effects in other cell types indicating the importance of tissue context for p53-mediated modulation of tumor cell sensitivity to treatment.

1524

### p53 Forms a Disulfide Bond Between Cys275 and Cys277 upon Treatment with Oxidants: A Possible Mechanism for Regulation of DNA Binding Activity

J. J. Leon,[1] H. Wu,[1] C. Vinci,[1] J. Whitelegge,[2] K. Mann,[3] J. Momand[1]; [1]Chemistry and Biochemistry, California State University Los Angeles, Los Angeles, CA, [2]Molecular Sciences, University of California, Los Angeles, CA, [3]Biological Sciences, University of Alaska, Anchorage, AK

Background: p53 tumor suppressor binds to a consensus sequence to regulate transcription of genes. Treatment of p53 with agents that oxidize cysteine thiols prevents it from binding to DNA. Objective: Map the site(s) of oxidation and determine the molecular structure of the oxidized thiol groups. Method: Plasmids expressing *p53* mutants with serine codons substituted for each of nine conserved cysteine codons in the DNA binding domain were transfected into mammalian cells and treated with the p53 oxidizing agent, pyrrolidine dithiocarbamate. Mutants that failed to oxidize were determined. To confirm that p53 could be oxidized at these positions, air oxidized, recombinant wild-type p53 was modified with a

carboamidomethylfluorescein group (CF) at oxidized thiols. Trypsin-released peptides were analyzed by MALDI mass spectrometry to determine if observed masses matched CF-conjugated cysteine-containing peptides. The structure of the oxidized cysteine thiols was determined by microliquid chromatography tandem mass spectrometry (µliquid CTMS) analysis of trypsin-released released peptides. Results: p53 with substitutions at position 275 and 277 were substantially inhibited from oxidation compared to other mutants. CF was bound to Cys275 or Cys277, and Cys182. µliquid CTMS analysis showed evidence for the existence of peptide containing a disulfide bond between Cys275 and Cys277. Conclusion: When p53 is bound to DNA, the Cys277 thiol normally donates a hydrogen bond to the $NH_2$ group on cytosine located in the third position within the p53 consensus response element, (A/T)GPyPyPy. It is proposed that the donating hydrogen bond is removed by disulfide formation between Cys277 and Cys275 and that this oxidation event prevents p53 from binding to its consensus sequence.

1525

**BRCA1 Deficiency Mediates Paclitaxel Resistance through Premature Inactivation of Spindle Checkpoint in Breast Cancer Cells**

C. Chabalier, C. Lamare, C. Racca, M. Privat, A. Valette, F. Larminat; LBCMCP, UMR 5088 CNRS, Toulouse, France

BRCA1 germline mutations predispose women to early onset, familial breast and ovarian cancer. Despite its role in maintenance of genomic integrity, transcriptional regulation and ubiquitination, the exact mechanism of tumor suppression by BRCA1 remains to be defined. A few recent studies have implicated BRCA1 in the cellular response to agents that disrupt the mitotic spindle, including paclitaxel. In this study, we sought to determine the mechanism through which BRCA1 might modulate the paclitaxel response in cancer cells. Since it was recently shown that loss of spindle assembly checkpoint increases paclitaxel resistance (Sudo et al, 2004), we hypothesized that BRCA1 might regulate the mitotic checkpoint machinery. To test this possibility, we significantly reduced BRCA1 protein levels using siRNA in MCF-7 breast cancer cells and investigated the paclitaxel induced-spindle checkpoint activation. Using flow cytometry-based detection of phosphorylated histone H3 for the quantitation of mitotic cells, we showed that suppression of BRCA1 significantly reduces the paclitaxel-induced arrest at mitosis. Next, we showed that BRCA1 deficiency triggers premature cyclin B1 degradation and decrease in Cdk1 activity following paclitaxel treatment in MCF-7 cells, suggesting that BRCA1 downregulation results in premature activation of the anaphase-promoting complex. These findings were confirmed by showing that BRCA1 down-regulation induces premature sister-chromatid separation in MCF-7 cells following spindle damage, as determined by metaphase spreads analysis. Finally, measuring the cell viability, we found that MCF-7 cells transfected with BRCA1 siRNA display a significant increase in resistance to paclitaxel compared with the control cells. All together, our findings support the notion that down-regulation of BRCA1 expression mediates paclitaxel resistance through premature inactivation of spindle checkpoint in breast cancer cells. They link BRCA1 to the mitotic checkpoint which plays an essential role in the maintenance of chromosomal stability.

1526

**BRCA2 Regulates Mitotic Checkpoint upon DNA Damage via BubR1 Acetylation**

H. Lee, E. Choi, D. Hwang, J. Park; Dept. of Biological Sciences, Seoul National University, Seoul, Republic of Korea

Individuals with inherited mutations in BRCA2 are predisposed to early-onset breast cancers. Loss of Brca2 function in mice leads to defects in DNA double-strand break repair and progressive growth impediment. Tumorigenesis in these mice accompanies the inhibition of mitotic checkpoint, suggesting a crucial interplay between DNA repair machinery and mitotic checkpoint function in the maintenance of genetic integrity. Here, we provide the molecular mechanism of how BRCA2 regulates mitotic checkpoint upon DNA damage. BRCA2 acetylates BubR1 after DNA damage and inhibits ubiquitin-dependent proteolysis of BubR1. BubR1 level in BRCA2-deficient cells are significantly lower than wild-type, consistent with the observation that BRCA2-deficient cells possess aneuploidic population. An acetylation-defective BubR1 mutant, K250R, exhibits markedly lower protein level compared to wild-type. Taken together, these results suggest that BRCA2 links DNA repair and mitotic cell cycle arrest by acetylating BubR1 and increasing its stability upon genotoxic injury.

1527

**The Role of Znf217, a Gene Amplified in Breast Cancer, in Neoplastic Progression**

L. E. Littlepage,[1] P. Yaswen,[2] Z. Werb[1]; [1]Anatomy, University of California, San Francisco, San Francisco, CA, [2]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

The 20q13 region of the human genome is highly amplified in 20-30% of early stage human breast cancers (as well as in other cancers), and this amplification correlates with poor prognosis. The ZNF217 gene, which is a candidate oncogene in 20q13.2, is found as a component of the corepressor of transcription associated with the human histone deacetylase complex (CoREST-HDAC) and in a complex with the transcriptional co-repressor C-terminal binding protein (CtBP). Previous work has shown that overexpression of ZNF217 leads to immortalization of human mammary epithelial cell lines. To investigate the effect of ZNF217 on neoplastic progression, we cloned mouse Znf217 by PCR and characterized its role as a putative transcription factor. Using Gal4-fusion Znf217 constructs in transcription assays, we find that ZNF217 is a strong transcriptional repressor. We then inserted mouse Znf217 into a retroviral vector. Mouse mammary epithelial SCp2 cells infected with the mouse Znf217 retrovirus express the ZNF217 protein when induced, as determined by western analysis using an antibody raised against human Znf217 that we found cross-reacts with mouse Znf217. Cells overexpressing Znf217 reorganize their actin cytoskeleton and have altered nuclear morphology. Therefore we analyzed cell motility and migration using scratch wound healing assays in culture. SCp2 cells overexpressing Znf217 show increased motility in scratch assays in culture over 24 hours. These data suggest that repression of a transcriptional target by Znf217 might lead to increased motility of epithelial cells. Further characterization of downstream targets and expression patterns will elucidate the pathways that may be affected by ZNF217 in breast cancer progression.

1528

**Study of a Potential Breast Tumor Suppressor Gene, Int6**

J. Suo,[1] J. Sap,[2] E. Chang[1]; [1]Breastcenter, Baylor Colleage of Medicine, Houston, TX, [2]Department of Medicine, Cell Biology and Pharmacology, New York University School of Medicine, New York, NY

The int6 gene was first isolated in a screen using the mouse mammary tumor virus (MMTV) as an insertional mutagen to seek genes that are important for breast cancer formation. Reduction in Int6 expression is also frequently observed in human tumors. While the importance of Int6 and

breast cancer has been established in both mice and humans, its molecular function remains unknown. Our preliminary data derived from the study of an Int6 homolog in the fission yeast Schizosaccharomyce pombe support the hypothesis that Int6 regulates the function of the 26S proteasome, which functions to degrade growth regulatory proteins in a ubiquitin-dependent fashion. We envision that when Int6 functions are inactivated, the proteasome may be weakened to lead to accumulation of regulatory proteins, some of which are key players in the control of cell cycle and chromosome segregation. Therefore Int6 may acts as a tumor suppressor for breast cancer. To test this, we investigate whether altering Int6 functions can influence (1) proteasome functioning and (2) chromosome segregation in human cells, leading to transformation. We have identified several siRNA sequences that can efficiently knock-down int6 expression via direct transfection. For example, they knock down Int6 expression by approximately 80 and 50% in HeLa and MCF10A cells, respectively. Consistent with our yeast data, Int6-knocked-down HeLa cells frequently become multi-nucleated and contain lagging and missegregated chromosomes, suggesting that Int6 is important for chromosome segregation. We have raised a polyclonal antibody against Int6, which can immunoprecipitate Int6 from MCF10A and HeLa cells. We are currently investigating whether Int6 can co-immunoprecipitate proteasome subunits. Furthermore, we are using this and other Int6 antibodies to examine tumor tissues and cell lines by Western blots to determine whether Int6 expression may be altered during tumorigenesis.

1529

**PTEN Expression and Localization Affects Acini Formation of Human Mammary Epithelial Cells in Laminin-Rich Extracellular Matrix**

M. Fournier,[1] K. Martin,[2] K. Giaya,[2] I. Bosch,[2] M. Bissell,[1] P. Yaswen[1]; [1]Lawrence Berkeley National Laboratory, Berkeley, CA, [2]University of Massachusetts, Worcester, MA

**Background:** The growth arrest of non-malignant human mammary epithelial cells (HMEC) in laminin-rich extracellular matrix (lrECM) provides a three-dimensional (3D) model system in which to study a normal process of growth control that is disrupted in cancer. Approximately 50 percent of patients with breast cancer have a mutation in, or loss of, at least one copy of the PTEN gene, which results in the activation of PI3K signaling. It has been shown that inactivation of the PI3K pathway can cause partial or complete reversion of the malignant phenotype in the 3D model system. PTEN and the PI3K pathway are attractive candidates for integrators of signals from soluble growth factors, cell-matrix, and cell-cell interactions. **Hypothesis**: We hypothesize that PTEN and associated pathways perform pivotal roles in integrating the signals from cell-cell and cell-ECM interactions. **Methods and Results**: To systematically probe the molecular changes that lead to growth arrest and acini formation, we have analyzed HMEC expression profiles using microarrays. Transcripts exhibiting greater than 2-fold differences in 2 independent microarray experiments were analyzed by 1-way ANOVA as a function of time. Genes displaying significant ($p<0.05$) differential expression were selected. PTEN was identified by pathway analysis as a key regulatory gene. We observed that PTEN inhibition by shRNA induced significant proliferation, demonstrating that PTEN is required for growth arrest in the 3D. To determine whether endogenous PTEN expression levels and subcellular localization change with disruption of cell-cell contacts, we used E-cadherin function-blocking antibodies in HMEC cultures in 3D. Blocking E-cadherin function inhibited PTEN accumulation at cell-cell contacts, suggesting that disruption of E-cadherin-mediated cell adhesion interferes with the PTEN-dependent growth suppression function.

1530

**The Centrosome-Associated Kinase hMps1 is Highly Expressed in Estrogen Receptor Negative Breast Cancers**

J. M. Colon-Franco,[1] M. A. Zschunke,[2] V. C. Negron,[2] A. Leontovich,[2] E. A. Perez,[3] W. L. Lingle[2]; [1]Tumor Biology, Mayo Clinic, Rochester, MN, [2]Experimental Pathology, Mayo Clinic, Rochester, MN, [3]Medical Oncology, Mayo Clinic, Jacksonville, FL

Human Mps1 is a centrosome-associated kinase that has been reported to function in centrosome duplication. Because centrosome amplification has been implicated in the origin of chromosomal instability during tumor development, we are investigating a possible role of Mps1 in breast cancer and centrosome amplification. We performed quantitative RT-PCR for Mps1 on a total of 95 breast tissues with known estrogen receptor (ER) and lymph node (LN) status. These tissues included 14 normal breast tissues from cosmetic reduction mammoplasties along with 27 ER+/LN-, 23 ER+/LN+, 18 ER-/LN-, and 13 ER-/LN+ tumors. Here, we report the correlation of Mps1 mRNA levels with the ER and LN status of the tumor tissues. The average expression level of Mps1 in ER+/LN- (0.158) and ER+/LN+ (0.201) tumors did not significantly differ from each other or from normal tissue (0.006). However Mps1 expression was significantly greater in ER-/LN+ (1.027) than in ER-/LN- (0.592) tumors, and both groups were significantly greater than normal tissue or ER+ tumors. These results are consistent with quantitative RT-PCR findings for Mps1 in breast cancer cell lines. Mps1 mRNA levels were low in hTERT-hMEC and MCF-7, normal and non-aggressive cell lines, respectively, compared to higher levels present in the more aggressive and invasive T47-D and MDA-MB-231 cell lines. These findings lead us to conclude that Mps1 mRNA is overexpressed in breast cancer cells and tissues with higher invasive potential that lack estrogen sensitivity. Our results suggest a role for Mps1 as a marker for breast cancer aggressiveness and as a potential target for therapy of breast cancers that may not be responsive to anti-estrogen therapies.

1531

**RC-RNase-induced Cytotoxicity in MCF-7 Cells is Modulated by Estrogen Receptor**

M. Hsu,[1] C. Wei,[2] J. Wang[3]; [1]Institute of Biology and Anatomy, NDMC, Taipei, Taiwan Republic of China, [2]Life Sciences, Fu Jen Catholic University, HsinChuang, Taipei, Taiwan Republic of China, [3]College of Medicine, Fu Jen Catholic University, HsinChuang, Taipei, Taiwan Republic of China

Several frog oocyte-derived antitumoral ribonucleases are noted these days, such as onconase from *Rana pipiens* and RC-RNase from *Rana catesbeiana*. RC-RNase belongs to the pancreatic ribonuclease A superfamily, is a pyrimidine-guanine sequence specific ribonuclease, which shares 48.2% amino acid sequence homology to onconase, which is a well-known anti-tumor drug and currently on its phase III trail in USA. RC-RNase processes cytotoxicity for varies tumor cells but rarely to normal cells such as fibroblasts. Our present studies demonstrated that RC-RNase is toxic to estrogen receptor (ER)-positive breast cancer cell lines, MCF-7 and ZR-75-1, but not to ER-negative cells, ZR-75-30 and MDA-MB-231. RC-RNase induced cell death through an apoptotic pathway, and blocked the expressions of ER protein, Bcl-2 and Bcl-$X_L$. We also found that the amount of ER mRNA expression accumulated in MCF-7 cells with a time- and dose-dependent condition. The ER protein expression in a selected RC-RNase-resistant MCF-7 cell line was higher, but, its mRNA level was uninfluenced. These results inferred that RC-RNase induced cytotoxicity of MCF-7 cells could probably act on the translation level to block the ER protein expression, which then is not able to keep the cells

alive. The results that RC-RNase activates caspase-3-like, caspase-8 and caspase-9 in MCF-7 cells indicated that mitochondria-mediated apoptosis mechanism could be involved. Over-expression of ER in MCF-7 cells by transfection of a plasmid, pCMV-sER-neo, rescued RC-RNase induced cytotoxicity. We concluded that RC-RNase-induced cytotoxicity in MCF-7 cells is modulated by ER.

1532

**SURF4 is Overexpressed in Gastric Cancers and Has an Oncogenic Potential During Tumorigenesis**

D. Lee, J. Chang, S. Song, S. Byun, D. Lim; Department of Biological Sciences, KAIST, Daejeon, Republic of Korea

SURF4(Surfeit 4) is located in the surfeit gene cluster, which is a conserved integral membrane protein containing multiple putative transmembrane regions. However, the biological role of SURF4 remains largely undefined. We found SURF4 gene is aberrantly overexpressed in human gastric cancers. SURF4 protein localize to endoplasmic reticulum and its overexpression accelerated G1-S transition. In addition, SURF4 exerted the ability to induce multiple focus formation and sustain anchorage-independent growth of transfected NIH3T3 cells. Moreover, NIH3T3 cells expressing SURF4 formed tumors in athymic nude mice. Therefore, SURF4 was sufficient to support anchorage-independent growth of NIH3T3 cells and tumor formation *in vivo*. Taken together, our observations suggest that overexpression of SURF4 induced tumorigenesis may have positive effects on tumor growth and metastatic progression.

1533

**Eps8 Upregulates the Expression of Focal Adhesion Kinase and Contributes to Human Colon Tumorigenesis**

T. Leu; Pharmacology, National Cheng Kung University, College of Medicine, Tainan, Taiwan Republic of China

Eps8, a common substrate for both receptor and nonreceptor tyrosine kinases, has been characterized as an oncoprotein and participates in Src-mediated transformation in murine fibroblasts. However, to date, its involvement in human cancers is still obscure. In this study, we observe the overexpression of Eps8 in both human colon cancer cell lines and human colorectal tumor specimens. Interestingly, as compared to cells with low Eps8 expression (i.e. SW480), increased growth rate is detected in cells with high Eps8 expression (i.e. SW620 cells), implicating the critical role of Eps8 in colon cancer formation. Indeed, reduced cell proliferation in culture and tumor growth in nude mice is observed in SW620 cells bearing *eps8*-siRNA. Surprisingly, a statistically strong correlation between the expression of Eps8 and FAK is observed in human colon tumor specimens and the capacity of Eps8 to modulate FAK abundance is further confirmed. In agreement with its role in mitogenesis, ectopical expression of FAK in cells with *eps8*-siRNA restored cellular growth. We thus propose that Eps8 overexpression in human colon tumors facilitates proliferation by upregulation of FAK.

1534

**Transcriptional Inhibition of Cyclin D1 by HIPK2: A Novel Tumor Suppressing Mechanism through LEF1/beta-catenin Complex**

G. Wei, S. Ku, G. Ma, A. A. Tang, E. J. Huang; Pathology, UCSF & VA Medical Center, San Francisco, CA

Transcriptional control of cyclin D1 expression plays important roles in cell cycle progression. It is well-established that beta-catenin interacts with TCF/LEF-1 at the enhancer elements of cyclin D1 and positively regulates its transcription. Disruption of such mechanism has been implicated in tumorigenesis in multiple organs. Here we provide evidence that transcriptional corepressor homeodomain interacting protein kinase 2 (HIPK2) is a tumor suppressor that negatively regulates cyclin D1 expression. Loss of HIPK2 leads to a selective increase in the transcription of cyclin D1. As a consequence, HIPK2 null MEFs show rapid G1-S transition, increased BrdU incorporation, and increased colony formation. Conversely, over-expression of HIPK2 inhibits beta-catenin-mediated activation of cyclin D1 transcription and induces growth arrest. The mechanism by which HIPK2 suppresses cyclin D1 transcription does not involve phosphorylation or accelerated degradation of beta-catenin. Rather, HIPK2 regulates cyclin D1 transcription by forming a protein complex with LEF1 and beta-catenin, and presumably by recruiting other transcriptional repressors. Since cyclin D1 is a potent oncogene, the inhibitory effects of HIPK2 leads us to hypothesize that HIPK2 may function as a tumor suppressor. Indeed, our data show that HIPK2 is expressed in transient amplifying cells and a small number of stem cells in the epidermis. In a skin tumorigenesis paradigm, loss of one or both alleles of hipk2 leads to an increased propensity of papilloma formation with most of the tumors progressing into carcinoma. Taken together, our data underscore the important role of HIPK2 as a haploid insufficient tumor suppressor that negatively regulates cell growth and proliferation by inhibiting cyclin D1 transcription. These results further suggest that HIPK2 could be a potential therapeutic target for cancer chemotherapy.

1535

**Differential Effects of Stat3 Inhibition in Sparse vs Confluent Cells**

A. Anagnostopoulou,[1] A. Vultur,[1] R. Arulanandam,[1] J. Turkson,[2] R. Jove,[2] L. Raptis[1]; [1]Pathology and Molecular Medicine, Queens' University, Kingston, ON, Canada, [2]Moffitt Cancer Center, Tampa, FL

We recently demonstrated a dramatic increase in Stat3 phosphorylation at tyr705 (Stat3-ptyr705) and Stat3 activity with cell density in normal cells, which peaked at approximately 1-2 days post confluence and gradually declined as cell death ensued. This observation hints at the possibility that Stat3 might have different targets in sparse vs confluent cultures. To examine such a mechanism, it is important to evaluate the effect of Stat3 inhibition at a precise time-point and this was previously attempted using several approaches: We previously reported that PY*LKTK, the putative Stat3 SH2 domain-binding sequence, coupled to a membrane-translocation sequence can disrupt Stat3:Stat3 dimer formation and subsequent Stat3 DNA-binding activity in intact cells. Our later results also demonstrated that the tripeptides PY*L and AY*L, and peptidomimetics designed using AY*L and PY*L as leads with a substitution of the Y-1 prolyl (or alanyl) residue by aromatic groups, are also strong disruptors of Stat3 DNA binding activity *in vitro* and in cultured cells. We also employed the platinum compounds, $PtCl_4$ and CPA-7, which can be effectively taken up by the cell. To achieve the instant introduction of the peptidimimetics we used a technique of *in situ* electroporation we developed, where an electric pulse is applied as the cells are growing on a conductive and transparent support of indium-tin oxide. In this setup, the electric pulse allows the instant introduction of the material with minimal damage to the cell, presumably because the pores reclose instantly, so that the cellular interior is restored to its original state. Our results using both approaches demonstrate that Stat3 inhibition at precisely the time of its peak activity post-confluence induces apoptosis, which is more pronounced in transformed cells, while Stat3 inhibition in normal, subconfluent cells induces merely a growth retardation.

1536

**Oncogenic Potential of Recombinant hERG: Effects on Growth and Role of Functional hERG Current**

D. M. Pier, J. R. A. Challiss, J. S. Mitcheson; Cell Physiology and Pharmacology, University of Leicester, Leicester, United Kingdom

Human ether-a-go-go related gene product (hERG) is a potassium channel found ubiquitously in embryonic tissue, but only in key excitable tissues in the adult. Cell transformation has been associated with hERG re-expression, and the related EAG channel has been shown to possess oncogenic potential (Pardo *et al*., EMBO J. **18**:5540-5547, 1999). Here we have investigated the effect of stable, recombinant hERG expression in NIH-3T3 cells. At physiological hERG expression levels there was no significant difference ($p=0.77$) in the proliferative rate or serum dependence of growth, compared to mock transfected cells. However, hERG expression increased the ability of confluent cells to overgrow each other resulting in a significant increase ($p=0.015$) in post-confluent cell density compared with wild-type cells. In contrast to the parental cell line, hERG-expressing NIH-3T3 cells grew in soft agar. hERG expression also significantly increased the rate of migration of NIH-3T3 cells into a wound (by 73%; $p=0.005$) compared to wild-type. hERG expression had no effect on the actin cytoskeleton of NIH-3T3 cells in sub-confluent cultures, however, at confluency evidence of cytoskeletal reorganization could be seen, corresponding with a rounding up of the cell to produce a more spindle-like appearance. None of the effects of hERG expression were affected by the presence of the hERG-blocking compound, dofetilide. In conclusion, stable expression of hERG produces some characteristics of a transformed phenotype in NIH-3T3 cells. Pharmacological evidence suggests that this effect on cell phenotype is not dependent on functional channel current. Supported by the British Biotechnology and Biological Sciences Research Council and GlaxoSmithKline.

1537

**Regulation of G1 Transcription by the Evi5 Oncogene**

E. W. Verschuren, A. G. Eldridge, A. Loktev, P. K. Jackson; Pathology, Stanford University, Palo Alto, CA

The timely degradation of cell cycle regulators by ubiquitin-dependent degradation is crucial for irreversible cell cycle progression. At G1/S these mechanisms are superimposed on inactivation of Rb family transcriptional repressor complexes by G1 cyclin/Cdk activity such that E2F-dependent transcription ensues. The Emi1 protein is an E2F target gene that functions to inhibit the anaphase-promoting complex (APC) ubiquitin ligase. Emi1 expression thereby allows for the accumulation of mitotic regulators including cyclins A and B. Upstream of Emi1, we recently identified the Evi5 oncogene as a factor that binds Emi1 and stabilizes Emi1 protein. Here, we studied the effect of Evi5 protein on underlying E2F transcriptional activation. Northern analysis shows that the abundance of Evi5 mRNA is not cell cycle regulated, as opposed to significant increases in Emi1 mRNA in late G1. Notably, Evi5 protein accumulation occurs just before the induction of Emi1 mRNA. Knockdown of Evi5 protein by siRNA treatment from synchronized cells results in a reduction of Emi1, cyclin A2 and cyclin E1 transcript levels as measured by Northern and RT-PCR analysis. These data suggest that Evi5 is a positive regulator of E2F-dependent transcription. We identified several SWI/SNF complex members as Evi5 interacting proteins using a yeast two-hybrid screen. This interaction was confirmed by co-immunoprecipitation of overexpressed as well as endogenous proteins. Interestingly, active Rb-mediated repression of cyclin E, A and D1 promoter is thought to require SWI/SNF remodeling. We are now studying the physiological relevance of Evi5 regulation of E2F transcription on G1/S progression.

1538

**Transforming Growth Factor-β Suppresses Telomerase Expression through the Proximal Region of the hTERT Promoter in a Smad-independent Manner**

A. Lacerte, S. Lemay, J. Lebrun; Hormones & Cancer Research Unit, McGill University, Montreal, PQ, Canada

Tumors develop when barriers that inhibit cell growth are overcome. One such barrier is the length of the telomeres. In 90% of cancers, the telomere length is maintained by the enzyme telomerase. The expression of hTERT, the protein component of telomerase, is closely associated with telomerase activity and is increased in most cancer cells. Transforming growth factor-β (TGFβ), a well-known inhibitor of cell proliferation, has been recently shown to decrease hTERT expression. The aim of the present work is to understand how TGFβ inhibits hTERT expression at the transcriptional level. To test this, we cloned the 2.0kb promoter region of the hTERT gene in front of the luciferase gene. In the keratinocyte HaCaT cancer cell line, we showed that TGFβ inhibits hTERT expression and hTERT promoter activity by 80%. Using an Exonuclease III approach, we truncated the promoter and performed luciferase assays with these deletion mutants. TGFβ repressed hTERT promoter activity through the -252 to +3 region. As TGFβ classically signals through the Smad pathway, we next evaluated its involvement using the inhibitory Smad7 or dominant negative (DN) Smad2 and Smad3 that fail to be phosphorylated. Smad7 overexpression reversed TGFβ-mediated decrease of hTERT promoter activity. Surprisingly, DN Smad2 or DN Smad3 did not reverse TGFβ's inhibition of hTERT promoter. Our data rather indicated that the Erk1/2 and p38 kinases pathways play direct role in TGFβ regulation of the hTERT promoter. Therefore, TGFβ decreases expression of the hTERT gene in addition to its known inhibitory effect on cell division, thus linking two mechanisms that suppress tumor development. Understanding the mechanism by which hTERT gene is upregulated in cancer cells suggests new avenues for the development of cancer therapy.

1539

**Inhibition of Cancer Cell Proliferation by Mutant Template Telomerase RNA Requires the Catalytic Action of Telomerase at Telomeres**

A. Goldkorn, E. H. Blackburn; Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA

Cancer cells contain telomerase activity that sustains their growth. A gene expression construct (MT-hTER/siRNA) was developed that knocks down human endogenous wild type telomerase RNA (wt-hTER) through the use of short interfering RNA (siRNA) and, at the same time, expresses a mutant-template hTER (MT-hTER) to take the place of the endogenous wt-hTER. This combination works synergistically to radically inhibit cancer cell line proliferation (1), but the intracellular mechanism by which this is achieved has not been demonstrated. To elucidate the manner in which the MT-hTER/siRNA construct exerts its inhibitory effects on human cancer cells, three variants of the human telomerase catalytic subunit (hTERT) were ectopically over-expressed via retroviral infection in the LOX human melanoma cell line - wild type (WT-hTERT), "dominant negative" enzymatically inactive (DN-hTERT), and the putative non-telomere-localizing (N125A+T126A-hTERT). MT-hTER/siRNA and vector control were ectopically expressed via lentiviral infection in LOX cells over-expressing each of the three hTERT variants, and the cells were assayed for proliferation and formation of DNA damage foci indicative of telomerase uncapping. Upon ectopic expression of MT-hTER/siRNA,

LOX cells over-expressing WT-hTERT developed significantly greater numbers of DNA damage foci and overall growth inhibition than LOX cells over-expressing the DN-hTERT or N125A+T126A-hTERT variants. Hence, MT-hTER/siRNA requires the presence of WT-hTERT - an enzymatically active, telomere-localizing telomerase catalytic subunit - in order to exert its cellular effects. These results provide direct evidence that supports a model by which MT-hTER/siRNA exerts its toxic effect through combining with endogenous hTERT to form a catalytically active RNP that adds incorrect tandem repeats to the telomere ends. MT-hTER/siRNA's mechanism of action may be exploited for telomerase targeting in cancer therapeutics. 1. Li, S. et al. Cancer Research 2004.

1540

**Substrate Binding of Eukaryotic Chaperonin TRiC/CCT is Mediated by Specificity and Plasticity**

C. Spiess, J. Frydman; Department of Biological Sciences and BioX Center, Stanford University, Stanford, CA

Chaperonins are multimeric cylindrical protein complexes designed to accommodate unfolded proteins in their inner chamber to assist their folding. The eukaryotic Group II chaperonin TRiC/CCT is in contrast to the homo-oligomeric Group I chaperonins, such as the prokaryotic chaperonin GroEL, a hetero-oligomeric complex composed of 8 paralog subunits. Heterogeneity of the subunits raises the question if the chaperonin utilizes this diversity for substrate recognition or mechanistic reasons. We addressed the question of substrate recognition with the Von Hippel Lindau tumor suppressor protein (VHL) as a model substrate. VHL is an obligate TRiC/CCT substrate and the binding determinants are well defined within two short hydrophobic beta-strands (1). Utilizing synthetic peptides spanning the two TRiC/CCT binding motifs of VHL we investigated with a combined cross-linking and mass-spectrometry approach if the paralog subunits show unique and specific binding patterns. We show that a discrete subset of TRiC/CCT subunits binds the same peptide. An identical binding specificity is observed in constructs comprising the isolated substrate binding apical domain of the subunits, showing that the binding specificity is provided independent of the context of other subunits in the complex. This expresses that the subunit heterogeneity of the TRiC/CCT complex is utilized for substrate specificity of the subunits, and allows the eukaryotic chaperonin to expand the mechanism of folding beyond the capabilities of a simpler homo-oligomeric complex. Nevertheless partially overlapping binding specificity suggests that recognition of sequences is still modular and the TRiC/CCT complex shows plasticity to accommodate the same determinant multiple times. (1) Feldman D, et al.(1999) Formation of the VHL-Elongin BC tumor suppressor complex is mediated by the chaperonin TRiC. Mol. Cell 4, 1051-1061.

1541

**Regulation of Nm23-H1 and Macrophage Migration Inhibitory Factor (MIF) by Direct Interaction**

H. Ha, H. Jung, H. Seong, T. N. Lam; Biochemistry, Chungbuk National University, Korea, Cheongju, Republic of Korea

Nm23-H1 is a putative metastasis suppressor and a member of Nm23/nucleoside diphosphate (NDP) kinase gene family. Screening of interacting proteins that potentially modulate Nm23-H1 activity through the yeast two-hybrid assay revealed that macrophage migration inhibitory factor (MIF) could interact with Nm23-H1. MIF was found to form *in vivo* complexes with Nm23-H1 in mammalian cells, as demonstrated by *in vivo* binding assay and coimmunoprecipitation experiments. The use of Nm23-H1 mutants (C4S, C109S, and C145S) and MIF mutants (C57S, C60S, C81S) revealed that this interaction was significantly affected by C145S Nm23-H1 mutant and C60S MIF mutant, but not other Nm23-H1 and MIF mutants, suggesting that a disulfide involving $Cys^{145}$ of Nm23-H1 and $Cys^{60}$ of MIF is responsible for Nm23-H1:MIF complex formation. In addition, this association was dependent on the reducing conditions such as dithiothreitol or β-mercaptoethanol but not in the presence of $H_2O_2$. We show that the interaction of Nm23-H1 with MIF is functional because cotransfection of MIF significantly inhibits Nm23-H1 activities such as autophosphorylation, NDP kinase activity, and phosphotransferase activity, whereas the coexpression of Nm23-H1 stimulates the D-dopachrome tautomerase activity of MIF in a dose-dependent manner. Moreover, Nm23-H1 stimulated p53-dependent transcription and, consistently, down-regulation of the endogenous Nm23-H1 by the transfection of the small-interfering RNA (siRNA) resulted in a decrease of p53-dependent transcription.

## Cell Cycle Controls I (1542-1562)

1542

**A Model for Epigenetic Therapy for IVF Embryos with Imprinting Errors**

O. Chami, T. Stojanov, D. Leigh, R. P. S. Jansen; R & D, Sydney IVF, Sydney NSW, Australia

Introduction: Manipulation and culture of embryos *in vitro* can lead to stresses different to those found *in vivo*. Recently, a suspected increase in the incidence of the epigenetic form of the Beckwith-Wiedemann syndrome has been associated with embryos produced by IVF. In epigenetic syndromes of this kind the abnormal phenotype is a result of the absence or altered regulation of the imprinted maternal or paternal gene. In this study, we used agents that alter DNA methylation and raise the possibility of their use as potential epigenetic therapies for embryos. Methods: Mouse embryos cultured from the 1 cell stage for 24 to 120 h and undifferentiated human embryonic stem cells developed from blastocyst outgrowths were used. Methylation status was assessed after treatment with 5-aza-2-deoxycytidine or no treatment. Extracted DNA was bisulfite modified using the MethylEasy kit (Human Genetic Signatures, Australia) and then subjected to methylation specific PCR for genes associated with cell cycle regulation, embryonic metabolism and epigenetic syndromes. Results: DNA methylation profiles could routinely be defined using 5-7 cells or from the equivalent of half an embryo. hES cell lines showed a normal methylation pattern for the selected genes. Cell treatment with 5-aza-2-deoxycytidine induced demethylation of the DNA within 48 h in mouse embryos and human ES cells. **Conclusions**: Analysing the methylomic state of preimplantation embryos might enable the determination of both the developmental status and the potential of an embryo. In the medium term, the use of DNA methylation and demethylation agents could assist in reprogramming stem cells or embryos produced by somatic cell nuclear transfer in the development of stem-cell based therapies. In the longer term, it might be possible to safely correct or to optimize embryonic and fetal developmental potential without altering the genetic code.

1543

**Proteomic Screen for Novel Mitotic Substrates of DNA-PK**

L. J. Jeffers,[1] S. Mohommad,[2] O. N. Jensen,[2] S. P. Jackson,[3] C. Morrison[1]; [1]Department of Biochemistry and NCBES, National University of

Ireland, Galway, Galway, Ireland, [2]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense University, Odense, Denmark, [3]Welcome Trust and Cancer Research UK Institute of Cancer and Developmental Biology, Cambridge, United Kingdom

The DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine kinase and a central player in the maintenance of genomic stability in eukaryotic cells. DNA-PK is primarily involved in the Non Homologous End Joining pathway for the repair of double strand breaks in damaged DNA but also serves in other diverse roles such as telomere capping and apoptosis. The aim of this study is to identify *bona fide* physiological DNA-PK chromosomal substrates. Metaphase chromosomes were isolated from mitotically arrested HeLa S3 cells and incubated with purified human DNA-PK in the presence of radioactive ATP. Mass spectrometry analysis of phosphorylated bands excised from one dimensional polyacrylamide gels identified potential novel chromosomal substrates. Certain putative substrates for DNA-PK were identified from this initial screen and this was followed up by the cloning, expression and *in vitro* phosphorylation of the purified recombinant candidate proteins. Our study identified the proteins Borealin and RuvB as potential *in vivo* phosphorylation targets of DNA-PK by mass spectrometry and *in vitro* analysis. However, site directed mutagenesis on the conserved TQ residues of both proteins did not abrogate the phosphorylation signal and liquid chromatography mass spectrometry did not reveal any specific phospho-peptide residues. Currently, we are continuing the screen for more potential mitotic substrates from the initial mass spectrometry data.

1544

## MAD1 Haplo-insufficiency and Tumor Development in Mice

Y. Iwanaga,[1] A. Miyazato,[1] Y. Chi,[1] K. Haller,[1] J. M. Ward,[2] K. Jeang[1]; [1]NIAID, NIH, Bethesda, MD, [2]NIAID, NIH, Rockville, MD

The mitotic spindle assembly checkpoint monitors attachment of chromosomes to spindle poles and ensures proper chromosome segregation of in mitosis. Mitotic arrest deficient protein 1 (MAD1) locates at kinetochore in prometaphase and is involved in activation of mitotic spindle assembly checkpoint. Down regulation of MAD1 is observed in some human cancer cells; however, the relationship of this to inactivation of the spindle checkpoint, aneuploidy, and tumorgenesis is unclear. We have created a knock out mouse model to examine the physiologic function of MAD1. Complete knock out of MAD1 ($MAD1^{-/-}$) is embryonic lethal. We have examined MAD1 haploinsufficient ($MAD1^{+/-}$) mice in aging studies conducted over 18 months and have observed a higher incidence in tumorgenesis ($p<0.05$) compared to wild type littermates. To ask whether perturbation of spindle assembly checkpoint would provoke tumorgenesis, we treated wild type and $MAD1^{+/-}$ mice with vincristine, a drug that inhibits spindle polymerization. We found a higher proclivity in $MAD1^{+/-}$ mice compared to wild type controls to developing lung adenoma ($p<0.03$) in response to treatment with vincristine. Finally, when we passaged $MAD1^{+/-}$ mouse embryonic fibroblast in athymic nude mice, we observed a markedly elevated incidence of fibrosarcoma, not seen with wild type control MEFs. Altogether, these results hold similarity with previously described $MAD2^{+/-}$ mice, and suggest a parallel function for MAD1 in regulating mitotic checkpoint and tumorgenesis.

1545

## The Role of p63 in Human Corneal Epithelial Cell Proliferation

D. M. Robertson, W. M. Petroll, H. D. Cavanagh; Ophthalmology, UT Southwestern Medical Center, Dallas, TX

PURPOSE: p63 has been reported as a corneal epithelial stem cell marker. This study evaluated p63 expression in human corneal epithelial cells in vivo and in vitro throughout the cell cycle. METHODS: p63 expression was assessed using polyclonal antibodies to p63 in normal human cornea and hTERT-immortalized human corneal epithelial cells by IF and Western blotting. Blocking peptides were used to confirm antibody specificity. To assess changes in p63 with the cell cycle, hTCEpi cells were double-labeled with antibodies to Ki-67 and p63. Whole mount human corneal tissue was used to establish patterns of p63 expression in vivo. All images were obtained with a laser scanning confocal microscope. To assess the regulatory role of p63 in the cell cycle, siRNA directed against p63 was evaluated at days 2 and 4 by double-labeling with p63 and Ki67. RESULTS: In normal human tissue, p63 was seen in all basal and wing cell layers, with a loss of expression seen only in superficial epithelial cells. In organotypic culture, p63 was seen at all stages of stratified epithelial development. Double-labeling with Ki-67 and p63 demonstrated no changes in the levels of p63 expression during mitosis and no changes in cell cycle regulation following 2 and 4 days siRNA. CONCLUSIONS: The localization of p63 in the human cornea in vivo and in vitro suggests that p63 is not a stem cell specific marker, but is present in differentiated cells arising from a stem cell lineage. Ki67 results suggest that p63 is not a cyclin and does not play a direct role in cell cycle regulation. Taken together, these data suggest that p63 is not a specific marker of proliferating cells and is not directly involved in regulating corneal epithelial proliferation.

1546

## Characterization of Early Growth Response Extracted from Human Pulpal Fibroblasts

I. Tamura,[1] B. Chaqour,[2] P. S. Howard,[3] T. Ikeo,[1] E. J. Macarak[3]; [1]Biochemistry, Osaka Dental University, Hirakata, Japan, [2]Anatomy and Cell Biology, State University of New York, Brooklyn, NY, [3]Anatomy and Cell Biology, University of Pennsylvania, Philadelphia, PA

The early growth response (EGR) gene family encodes for transcription factors that are implicated in regulating cell proliferation. There is little information on their expression in oral tissues. We investigated the expression of EGR-1 and -2 mRNAs and proteins in human pulpal fibroblast (PUF) using Western blotting, Northern blotting and FITC-labeled immunocytochemical techniques. Primary cultures of PUFs were obtained from three patients with informed consent. After cultivating PUFs in Dulbecco's modified Eagle medium (DME) for two days without fetal bovine serum (FBS), we incubated them in DME supplemented with 10% FCS for 15, 30, 45, 60 and 120 min. Measurement of 5-bromo 2'-deoxyuridine incorporation was highest in PUFs treated with FBS for 45 min. Expression of EGR-1 mRNA and protein were initially seen beginning 15 min after treatment and then EGR-1 protein subsequently gradually increased. Additionally, phosphorylation of EGR-1 was observed by immunoprecipitation using an antibody to EGR-1. Anti-EGR-1 antibody abundantly localized within the nuclear region 30 min after treatment. Expression of EGR-2 mRNA was greatest at 15 min after treatment while maximal expression of EGR-2 protein was observed at 60 min. By immunocytochemistry, EGR-2 localized in and around the nucleus after 45 min. These results demonstrate that in human pulpal fibroblasts, native and phosphorylated EGR-1 and EGR-2 mRNA and protein expression are strongly stimulated by FBS and that the EGR family plays a potentially important role in the regulation of cell activities such as growth.

1547

**Artificial Control of Cell Proliferation Using an N-terminal Domain of Simian Virus 40 Large T Antigen by Means of PEI-cationization**

H. Murata, J. Futami, M. Futami, M. Kosaka, H. Tada, M. Seno, H. Yamada; Okayama University, Okayama, Japan

Cationization with polyethylenimine (PEI) is a powerful strategy for protein transduction into cells. Protein transduction has an advantage over gene transduction that the expression of the protein function within cells is transient. In this study, we attempted artificial regulation of cell proliferation by protein transuduction of the N-terminal domain (1-132 a.a.) of simian virus 40 large T-antigen (SVLT-N), which specifically inactivates retinoblastoma family proteins (pRb, p107, and p130), but not p53. In order to deliver SVLT-N into cells, we employed an indirect-cationization method as follows. First the SVLT-N was biotinylated and then mixed with PEI-cationized avidin (PEI-avidin). For the biotynylation of the SVLT-N, biotins were conjugated via disulfide bonds (biotin-SS-SVLT-N), which are cleavable in cytosolic reducing environments, because carrier domain of PEI-avidin might interfere the normal function of SVLT-N. To evaluate whether SVLT-N transduced in this way sufficiently drives the growth-arrested cells back into the proliferation stage, quiescent Balb/c 3T3 cells were treated with our new device (PEI-avidin/biotin-SS-SVLT-N) under the confluent condition. As a result, we succeeded to introduce the SVLT-N efficiently in the cells with an accumulation of the protein into their nuclei, in which the protein actually trapped cellular pRb. An assessment of BrdU incorporation also showed that the introduced protein caused a release from contact inhibition of the quiescent cells. In addition, some remarkable change in expression levels of cell cycle-related proteins (up-regulation of cyclin A and down-regulation of p27/Kip1) was detected in parallel with the intracellular contents of the SVLT-N. These results suggest that protein transduction of SVLT-N technology has great potential *in vitro* cell regulation and that may pave a new way for *ex vivo* cell expansion for cell therapy.

1548

**Early p38 Activation in C2C12 Myoblasts Maintains Myoblast Proliferation, While Later p38 Activation is Required for Fusion**

J. M. Churko, P. A. Merrifield; Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada

During myogenesis, multiple signaling pathways regulate the ability of myoblasts to withdraw from the cell cycle, elongate, fuse and express contractile protein isoforms such as myosin heavy chain (MyHC). The p38 mitogen-activated protein kinase pathway has been shown to both promote and inhibit myogenesis in C2C12 myoblasts cultured in vitro. This controversy may be the result of two temporally separated p38 activation events which are involved with different aspects of myogenesis (Weston et al. J Cell Sci. 116, 2885(2003); Cuenda and Cohen. J Biol Chem. 274, 4341 (1999)). To further examine the role of p38 activation in myogenesis, C2C12 myoblasts were initially grown in growth medium and phosphorylated p38 (p-p38) levels examined at 0 h, 1h, 6h, 12h, 24h, 36h, 48h, 60h, and 72h following the addition of differentiation medium. Western blot analysis with p38 and p-p38 specific antibodies demonstrated that p38 followed a biphasic pattern of phosphorylation, with an initial drop in p-p38 levels immediately following the addition of differentiation medium and increasing p-p38 levels after 36h. Inhibition of the alpha and beta isoforms of p38 with 10uM SB220025 in growth media decreased BrdU incorporation and cell counts indicating that p-p38 levels are required for myoblast proliferation. When the inhibitor was removed after 36h, myoblasts fused and expressed MyHC. However, adding the inhibitor after 36h blocked myoblast fusion. These results suggest that p-p38 levels are required to keep myoblasts in a proliferative state and that a subsequent activation of p38 phosphorylation following cell cycle withdrawal is required for myoblast fusion. (Supported by Discovery Grant 105869-02 RGPIN from NSERC).

1549

**Role of Cyclooxygenase-2 Glycosylation in Enzyme Turnover and Cell Proliferation**

M. B. Sevigny,[1] M. Hughes-Fulford[1,2]; [1]Laboratory of Cell Growth, Veterans Affairs Medical Center, San Francisco, CA, [2]University of California, San Francisco, CA

Cyclooxygenase-2 (COX-2) catalyzes the rate-limiting step in the biosynthesis of prostaglandins and thromboxanes by converting fatty acid arachidonic acid (AA) into prostaglandin $H_2$. Consequently, COX-2 plays an important role in various physiological as well as pathophysiological conditions, such as cancer. COX-2 exists as two glycoforms-- 72 and 74 kDa-- with the 74 kDa form resulting from an additional oligosaccharide chain at residue $Asn^{580}$. Since neither the biological significance nor the mechanism behind this differential glycosylation have been fully determined, the objective of this study was three-fold: (1) to determine if COX-2 glycosylation is affected by its substrate AA; (2) to elucidate the effect of glycosylation on COX-2 activity; and (3) to reveal any effects COX-2 glycosylation may have on cell division. Site-directed mutagenesis of the COX-2 gene at $Asn^{580}$ was accomplished, and transient transfection of COS-1 cells with either the mutant or wild-type gene was achieved. COX-2 glycoforms were analyzed via Western blotting, while activity was determined using an ELISA that measured the presence of the downstream product prostaglandin $E_2$. Finally, cell proliferation assays were performed on transfected and nontransfected cells. AA did not affect the expression of either the 72 or 74 kDa glycoforms. Cells transfected with the mutant COX-2 gene accumulated a greater amount of COX-2 protein-- expressed as the 72 kDa glycoform as well as an unexpected 70 kDa form-- and exhibited ~5-fold greater COX-2 activity compared to cells expressing both 72 and 74 kDa glycoforms. Cells unable to express the 74 kDa glycoform also tended to multiply at a significantly slower rate than cells expressing both glycoforms. In conclusion, this study demonstrated that COX-2 enzyme turnover depends upon glycosylation of the 72 kDa glycoform, and this glycosylation may subsequently affect the rate at which cells divide.

1550

**S-nitrosylation Affects Activity of E2F1**

K. Ravi,[1] N. Dyson,[2] G. Enikolopov[1]; [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, [2]Massachusetts General Hospital Cancer Center, Charlestown, MA

Nitric oxide (NO) has been implicated in the regulation of cell division, differentiation, and apoptosis in many cell types and several developmental settings. We have previously shown that during Drosophila development, NO exert part of its antiproliferative action through the Rb-E2F pathway (1, 2). E2F1 transcription factor is an important regulator of the cell cycle, affecting several cell cycle checkpoints; furthermore, it can induce apoptosis (3). We here studied the molecular mechanisms of the NO interactions with the Rb-E2F pathway. We used mouse brain microvascular endothelial (bEnd.3) cells, which produce large amounts of endogenous NO, as a model system to study the action of NO on E2F1. Using the biotin-switch method to detect S-nitrosylation of cysteine residues, we found that E2F1 is S-nitrosylated upon transfection into the bEnd.3 cells,

and that this modification can be suppressed by DTT and by NO synthase inhibitor L-NAME. To identify the residues that are modified by NO in the E2F1 molecule, we mutated each of the E2F1 cysteine residues to serines and found that two out of five residues are S-nitrosylated in bEnd.3 cells. These E2F1 mutants, as well as the double mutant, show altered transcriptional activity in the presence of Rb and DP, indicating the importance of the nitrosylation status of these two cysteine residues. We are now characterizing the interactions between the relevant E2F1 cysteine mutants and the E2F1-binding proteins to gain insights into the functional importance of the E2F1 modifications by NO. 1. Kuzin et al.,.Curr Biol., 10:459-62, 2000. 2. Stasiv et al., Genes&Dev., 18:1812-23, 2004. 3. Dimova and Dyson, Oncogene, 24:2810-26, 2005

1551

**5-Fluorouracil Mediates Apoptosis and $G_1$/S Arrest through a P53-independent Pathway in Laryngeal Squamous Cell Cancers**

H. C. Liu, G. G. Chen, A. C. Vlantis, C. A. van Hasselt; Department of Surgery, The Chinese University of Hong Kong, Hong Kong, China

5-Fluorouracil (5Fu) is commonly used as a chemotherapeutic agent in laryngeal cancer treatment. Cell cycle arrest or apoptosis induced by 5Fu can be either p53-dependent or p53-independent in various cancers but such a situation is unknown in laryngeal squamous cell cancer. In this study, we investigated the cytotoxic effect of 5Fu on laryngeal squamous cancer cell lines and evaluated the role of p53 in 5Fu treatment. We employed two human laryngeal squamous cancer cell lines with different p53 status - one (UMSCC12) has a truncated p53 and the other (UMSCC11A) a mutant but functional p53. Using cytotoxicity assay and Annexin V staining, we proved that 5Fu induced apoptosis of both cell lines in a dose- and time-dependent manner, suggesting that the process was p53 independent. 5Fu induced the accumulation of active pRb and $p21^{WAF1/CIP1}$ in both UMSCC12 and UMSCC11A. However, 5Fu did not activate p53 expression in either UMSCC12 or UMSCC11A cells. In addition, $G_1$/S phase cell cycle arrest was associated with the antiproliferative activity of 5Fu in both cell lines. In order to gain insight into the role of p53 in response to 5Fu in laryngeal squamous cell cancers, we further transfected a wild-type p53 plasmid or an empty pcDNA3.1 vector into UMSCC12 cells and found in both p53-transfected and vector-transfected cells, 5Fu increased p21 and pRb expression without significant accumulation of p53. Our result suggests that apoptosis and $G_1$/S cell arrest mediated by 5Fu in laryngeal cancers may be p53-independent but p21-dependent or p21-Rb-dependent. We also assume that p53 may not be the only target in controlling apoptosis and cell cycle arrest in laryngeal cancer treatment. Further study will need to examine the pathway of p21 and pRb in laryngeal cancer receiving chemotherapy.

1552

**Regulation of *dmyc* Transcription and Cell Cycle Progression by Hfp, Crol and Hay**

N. Mitchell, H. Richardson, L. Quinn; Cell Cycle and Development, Peter MacCallum Cancer Centre, Melbourne, Australia

We have shown that Half pint (Hfp), the *Drosophila* homologue of the mammalian *c-myc* transcriptional repressor (FIR), is required to inhibit cell cycle progression in *Drosophila*. Hfp negatively regulates developmentally coordinated cell cycles in *Drosophila* since (1) *hfp* mutant imaginal tissues undergo ectopic S phases and (2) overexpression of *hfp* inhibits cell cycle progression. Hfp negatively regulates two key cell cycle genes; (1) the *Drosophila* homolog of the *c-myc* cell growth regulator (*dmyc*) at the level of transcription and (2) the *Drosophila* homologue of the critical G2-mitosis regulator Cdc25 phosphatase (Stg) at the protein level. In an effort towards understanding the factors that work downstream of Hfp to regulate *dmyc* transcription and Stg/Cdc25 protein stability we have used a wing phenotype, resulting from overexpression of a *UAS-hfp* transgene with the *en-GAL4* driver, to screen for Hfp interactors. Overexpression of Hfp in the posterior of the wing imaginal disc results in fewer cell cycles and a reduced/disrupted adult wing phenotype, which is amenable to genetic modification. We have identified the Ecdysone responsive transcription factor *crooked legs* and the TFIIH helicase component *haywire* as genes which, when halved in dose, are capable of enhancing the Hfp wing phenotype. We will present evidence that suggests these factors act downstream of Hfp to positively regulate cell cycle progression.

1553

**Role and Regulation of the Cdc25b Phosphatase at the G2/M Checkpoint**

B. Ducommun; LBCMCP, CNRS, Toulouse, France

CDC25 are dual specificity phosphatases that are responsible for the dephosphorylation and the activation of CDK/cyclin complexes at key cell cycle transitions. Our presentation will discuss ongoing projects focusing on the spatial and temporal regulation of CDC25B, one of the three members of this family, and on its involvement in the control of the G2/M transition. We have shown that CDC25B is phosphorylated in vitro and in vivo by a number of protein kinases including Aurora-A, pEg3 and CHK1. The phosphorylated sites have been identified by mass spectrometry. Using specific antibodies, we have shown that these phosphorylated forms first localise at the centrosome level where they play an essential role in the regulation of CDC25B activity and the subsequent control of entry into mitosis. We will present experimental evidences indicating that these regulatory mechanisms are operating in a normal cell cycle and upon activation of the DNA damage activated checkpoint.

1554

**The szy-20 (bs52) Mutation is a Suppressor of zyg-1 and Defines a Novel Regulator of the Cell Cycle**

M. Song; LBG, NIH/NIDDK, Bethesda, MD

The centrosome serves as the primary microtubule-organizing center (MTOC) of the cell and at mitosis plays an important role in establishing a bipolar spindle. Previously a kinase, ZYG-1 was identified as a regulator of centrosome duplication in *C. elegans*. To enhance our knowledge of the biological process of centrosome duplication, we sought to identify additional factors that interact with ZYG-1 and/or are involved in the centrosome duplication process. This should allow us to formulate the genetic pathway(s) that regulate the centrosome during the cell cycle. Toward this end, we carried out EMS mutagenesis on a *zyg-1 (it25)* mutant strain and identified a mutation *bs52* that suppresses the embryonic lethality of the *zyg-1 (it25)* mutation. The bs52 mutation defines a gene that we call *szy-20* (for suppressor of *zyg-1*). *szy-20* encodes a novel coiled-coil protein. In addition to suppressing loss-of-function alleles of *zyg-1*, the *szy-20 (bs52)* mutation itself exhibits a strong temperature-sensitive embryonic lethality. We have begun phenotypic characterization of *szy-20 (bs52)* during the early cell cycles in C. elegans embryos. Immunostaining and video microscopy of *szy-20(bs52)* mutant strain reveals defects in polar body extrusion,cytokinesis and other aspects of cell cycle process. As expected in a double mutant strain of *szy-20 (bs52)* and *zyg-1 (it25)*, we have observed centrosome duplication at restrictive-temperature. We are continuing to analyze this gene molecularly and cytologically, by employing a variety of genetic and molecular tools to determine what the biological function of *szy-20* might be and to understand the nature of its interaction with *zyg-1*.

1555

**Phospho-regulation of Kinetochore Protein-protein Interaction and Chromosome Segregation by the Nek2a Kinase**

J. Yao, X. Cai, S. Pei, Y. Zhang, Z. Guo, J. Zhu, X. Yao, Y. Wang; Cell Cycle Control, University of Science & Technology of China, Anhui, China

Chromosome segregation in mitosis is orchestrated by protein kinase signaling cascades. A biochemical cascade named spindle checkpoint ensures the spatial and temporal order of chromosome segregation during mitosis. Our recent studies show that NEK2A possibly functions as a novel integrator of the spindle checkpoint signaling (J. Biol. Chem. 2004. 279, 20049). The fact that the phosphorylation of kinetochore protein Hec1 by Nek2 kinase is essential for faithful chromosome segregation (J Biol Chem. 2002 277, 49408) propelled us to delineate Nek2A-mediated phospho-regulation of kinetochore function. Using Hec1 as bait, our yeast two-hybrid screen has identified CENP-H as an interacting partner of Hec1 and mapped their interaction domains. The inter-relationship of Hec1-CENP-H was then validated by immunoprecipitation and pull-down assay using bacterial recombinant proteins. Both Hec1 and CENP-H are co-localized to kinetochore of mitotic cells while their interaction defines a stable kinetichore association and faithful chromosome segregation. Mass spectrometric analyses of Hec1 phosphorylated by Nek2A in vitro indicate that Ser165 is a major substrate. Interestingly, phosphorylation of Ser165 modulates Hec1-CENP-H interaction in vitro and chromosome segregation in yeast. The importance of Nek2A kinase in chromosome segregation was validated by a Nek2A depletion experiment in which Nek2A deficiency resulted a chromosome bridge phenotype with sister chromatid inter-connected. Currently, we are evaluating how Nek2A phospho-regulation of Hec1 governs chromosome stability in mitosis using real-time imaging.

1556

**The Mitotic Exit Network Mob1p-Dbf2p Kinase Complex Colocalizes with Cdc14p Phosphatase in the Nucleus and Functions at Kinetochores**

J. Stoepel,[1,2] M. A. Ottey,[1] C. Kurischko,[1] P. Hieter,[2] F. Luca[1]; [1]Dept. of Animal Biology, University of Pennsylvania, Philadelphia, PA, [2]Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada

The *Saccharomyces cerevisiae* mitotic exit network (MEN) is a conserved signaling network that coordinates CDK inactivation, cytokinesis and G1 gene transcription. Cdc14p phosphatase plays a pivotal role in MEN signaling and mediates mitotic exit by dephosphorylating Cdk1p substrates and promoting Cdk1p inactivation. Cdc14p is sequestered in the nucleolus prior to anaphase and is transiently released in early anaphase and later in telophase. It was previously established that Cdc14p regulates the localization of chromosomal passenger proteins, which redistribute from kinetochores to the mitotic spindle during anaphase (Pereira and Schiebel, 2003). The role of Cdc14p in passenger protein localization was proposed to be independent of other MEN proteins. Here we present cytological evidence that the MEN protein kinase complex Mob1p-Dbf2p partially localizes to the nucleus during mitosis, associates with kinetochores and colocalizes with Cdc14p. We confirmed the kinetochore associations of Mob1p, Dbf2p and Cdc14p by chromatin immunoprecipitation and established that the kinetochore associations are Ndc10p-dependent. To determine if Mob1p-Dbf2p contributes to passenger protein localization during anaphase, we analyzed the localization of Ipl1p, Sli15p and Bir1p (orthologs of mammalian Aurora kinase, INCENP and survivin) in conditional MEN mutants. Significantly, we found that both Mob1p and the Mob1p-Dbf2p activating kinase Cdc15p are required for maintaining chromosomal passenger proteins on the spindle during anaphase and telophase. Collectively, these data reveal kinetochores as sites for MEN signaling and implicate MEN in coordinating changes in passenger protein localization with mitotic exit.

1557

**Four-Dimensional Imaging of Mitotic Regulatory Proteins of Aspergillus nidulans**

T. Nayak, E. Szewczyk, C. E. Oakley, B. R. Oakley; Molecular Genetics, Ohio State University, Columbus, OH

A γ-tubulin mutation of *Aspergillus nidulans* affects the regulation of mitotic progression independent of microtubule nucleation (Prigozhina *et al.*, 2004, Mol. Biol. Cell 15:1374-1386). We hypothesize that this involves altered binding of mitotic regulatory proteins to the spindle pole body (SPB). In collaboration with the labs of Dr. Michael Hynes and Dr. Stephen Osmani, we have developed a rapid and efficient gene targeting procedure for *A. nidulans* that allows us to create GFP-tagged proteins easily. We have created C-terminal GFP-tagged versions of γ-tubulin, *bimA* (an APC component), and the *A. nidulans* homologs of Mad2, Cyclin B, Cdc14 and Mps1 and, as a first step in determining if their distribution is altered by γ-tubulin mutations, we have begun to 4D image them in a wild-type background. In each case the tagged gene is functional and is controlled by its normal promoter. In interphase, Cdc14 is in the nucleoplasm and at the SPB, but (unlike in *Saccharomyces cerevisiae*) it is largely absent from the nucleolus. As nuclei enter mitosis, Cdc14 disperses, then reappears on the spindle and then, in anaphase, at the SPBs. It becomes dispersed in the nucleoplasm again as the nuclei enter G1. Cyclin B is visible in the nucleoplasm and on the SPB of G2 nuclei. As nuclei enter mitosis, it is briefly located at the SPBs, but it disappears rapidly and is hard to detect by anaphase. Mad2 is found in multiple dots at the nuclear envelope in interphase. As nuclei enter mitosis it moves briefly to the SPB then is seen along the spindle. The nuclear envelope dots re-form as the nuclei exit mitosis. Supported by grant GM31837 from the NIH.

1558

**BubR1: A Checkpoint Protein also Required for Chromosome Congression**

A. Maia, C. E. Sunkel; Molecular Genetics of Mitosis, Institute for Molecular and Cell Biology, Porto, Portugal

During cell division, chromosomes must attach to microtubules and become bioriented to ensure proper segregation of the genome. The fidelity of this process is ensured by the spindle checkpoint. Mutations in genes involved in this surveillance mechanism were predicted to cause chromosome instability and aneuploidy, a hallmark of many cancers. Several proteins, including BubR1, participate in this checkpoint and are able to inhibit the ubiquitin ligase activity of the anaphase-promoting complex or cyclosome (APC/C). This prevents precocious sister chromatid separation and cyclins degradation until correct attachment/biorientation is achieved. Recently, a novel role for *h*BubR1 has been proposed in chromosome congression. Accordingly, we have analyzed this putative function of BubR1 in *Drosophila* S2 cells. We show that indeed depletion of BubR1 by RNAi causes abnormal congression associated with an increased number of chromosomes scattered along the spindle and/or at the poles. Furthermore, we find that blocking the metaphase-anaphase transition in BubR1 RNAi-treated cells with the proteasome inhibitor - MG132 - causes a severe increase in the number of chromosomes scattered along the spindle and at the poles. We conclude that the increase in the frequency

of this phenotype during extended inhibition of the proteasome is likely to be the result of inappropriate retention of Aurora B at kinetochores. An increased activity of Aurora B would then be responsible for the increasing destabilization of kinetochore-microtubule attachments leading to the higher frequency of congression defects observed in the absence of the proteasome inhibitor.

1559

**Emi1 Stably Binds and Inhibits the Core Anaphase Promoting Complex/Cyclosome as a Pseudosubstrate Inhibitor**

J. J. Miller, M. K. Summers, D. V. Hansen, M. V. Nachury, N. L. Lehman, A. Loktev, P. K. Jackson; Department of Pathology, Stanford University Medical School, Stanford, CA

Emi1 serves as an essential regulator of cyclin accumulation by inhibiting the activity of the Anaphase Promoting Complex (APC) from the G1-S transition, through S and G2 phases, and into mitosis. Here we show that Emi1 forms a stable complex with the APC to maintain the APC in the inactive state. To probe the Emi1-APC interaction, we used immobilized Emi1 beads, which efficiently capture the APC from cell extracts. A conserved destruction box and the zinc-binding region of Emi1 both contribute to the binding affinity and the efficiency of APC inhibition. We show that this high affinity interaction allows Emi1 to compete with the binding of D-box containing substrates to the APC. Mutation of the zinc-binding region converts Emi1 into a D-box dependent APC substrate, suggesting that Emi1 acts as a pseudosubstrate inhibitor. We will discuss the mechanism by which Emi1 binding to the APC core is regulated during mitosis, thereby allowing APC activation. We will also consider the role of the mitotic Cdc2 and Plk1 kinases in triggering Emi1 destruction and dissociation from the APC. We propose that combining a conserved degron with a high-affinity E3 binding domain provides a general model for how substrates of ubiquitin ligases can evolve to become regulated inhibitors.

1560

**The Evi5 Oncogene Regulates Cyclin Accumulation by Antagonizing Plk-Mediated Destruction of the Anaphase Promoting Complex Inhibitor Emi1**

A. G. Eldridge,[1] A. V. Loktev,[2] D. V. Hansen,[1] E. W. Verschuren,[2] J. D. R. Reimann,[2] P. K. Jackson[3]; [1]Cancer Biology, Stanford University, Stanford, CA, [2]Pathology, Stanford University, Stanford, CA, [3]Pathology and Microbiology & Immunology, Stanford University, Stanford, CA

The Anaphase Promoting Complex/Cyclosome (APC) inhibitor Emi1 controls progression to S phase and mitosis by stabilizing key APC ubiquitination substrates, including cyclin A. To identify factors upstream of Emi1, we inactivated Emi1-interacting proteins by RNA interference and have identified the Evi5 oncogene as a regulator of Emi1 accumulation. Evi5 antagonizes the polo-like kinase (Plk)- and $SCF^{\beta TrCP}$-dependent ubiquitination and destruction of Emi1 by binding to a site on Emi1 surrounding the DSGxxS degron. By blocking Plk-dependent phosphorylation of Emi1 and βTrCP binding, Evi5 functions as a stabilizing factor maintaining Emi1 levels in S/G2 phase. Evi5 protein is centrosomal, and is itself cell cycle-regulated, accumulating in early G1 following destruction of Plk1 and being triggered for ubiquitin-dependent proteolysis in early mitosis dependent upon Plk1 reaccumulation. Ablation of Evi5 results in precocious degradation of Emi1 by the $Plk/SCF^{\beta TrCP}$ pathway, causing premature APC activation, cyclin A destruction, cell cycle arrest, and uncoupling of the centrosome cycle, leading to mitotic catastrophe. We propose that the balance of centrosomally-localized Evi5 and Polo-like kinase activities determines the timely accumulation of Emi1 and cyclin, ensuring mitotic fidelity.

1561

**Medium Development for Thermostable Alfa-Amylase Production by *Bacillus Stearothermophilus* GRE1 Using Response Surface Methodology**

S. Hossain,[1] G. Haki,[2] S. K. Rakshit[3]; [1]Chemical Engineering, University of Calgary, Calgary, AB, Canada, [2]Debub University, Awassa, Ethiopia, [3]Food Engineering and Bioprocess Technology, Asian Institute of Technology, Pathumthani, Thailand

A culture medium for a newly isolated microorganism identified as *Bacillus stearothermophilus* GRE1 was optimized with regard to thermostable α-amylase production by means of conventional and response surface methods in a batch reactor.1.0 % (w/v) starch and 3.0 % (w/v) lactose were found to be optimal by single factor experiment for enzyme production. Mathematical model developed using response surface methodology also revealed that 1.02% starch and 3.12% lactose produced the best effect on the production of enzyme. A maximum concentration of 11.43 U/ml α-amylase was obtained under these medium constituents. In addition, enzyme production was growth-associated in this study.

1562

**Spatial Organization in Collective Movement of Epithelial Cells on a Collagen Gel Substrate**

H. Haga, K. Hayashi, K. Kawabata; Divsion of Biological Sciences, School of Science, Hokkaido University, Sapporo, Japan

Collective cell movement plays an important role in many physiological events, as it occurs throughout the processes of wound healing and early embryogenesis, such as gastrulation and invagination. However, the mechanism of the collective cell movement has not been elucidated. We had previously found that epithelial cells migrated collectively along one direction on a collagen gel substrate. Furthermore, we also found that there was a role assignment among epithelial cells on the collagen gel. The "leader" cells, which extended large lamellae and being accompanied by many "follower" cells, migrated in the direction of oriented collagen fibers. In the present study, in order to characterize the leader and the follower cells, we transfected MDCK cells with GFP-tagged myosin regulatory light chain (GFP-MRLC), and cultured them on type-I collagen gels. Temporal sequence of GFP-MRLC fluorescent images were captured by confocal laser scanning microscopy equipped with an incubational system. The leader cells had stress fibers and migrated like mesenchymal cells, whereas the typical follower cells did not have stress fibers and showed amoeboid-like migration. We also focused on the spatial distribution of phosphorylation of MRLC. Immunofluorescent observations stained with phosphorylated MRLC revealed that MRLC in a leader cell and the neighboring cells were highly phosphorylated, whereas MRLC in the follower cells were entirely dephosphorylated. These results suggest that mesenchymal-amoeboid transition occur in an epithelial colony on the collagen gels. In the annual meeting, we will present a probable model that the role assignment among epithelial cells may bring harmony into the cell society, and as a result, it may lead to the collective movement of the cells.

## Apoptosis II (1563-1589)

1563

### Induced Expression of a Pro-apoptotic Gene NALP5 Following Neuronal Injuries

C. Lo,[1] X. Ning,[1] C. Gonzales,[1] F. Liu,[1] E. M. Kajkowski,[1] M. M. Zaleska,[1] A. Wood,[1] J. S. Jacobsen,[1] B. A. Ozenberger[2]; [1]Wyeth Neuroscience, Princeton, NJ, [2]NHGRI, NIH, Bethesda, MD

With the emergence of apoptosis pathways as potential points of intervention in neurodegenerative disease, a strategy was developed to investigate the death domain protein repertoire of neurons. Using bioinformatics tools we identified several genes of interest that contain death domain such as CARD or Pyrin. We also sought to limit our searches to those genes that showed brain and/or neuronal localization. NALP5, a member of the NALP (NAcht Leucine-rich repeat Protein) family, represents one of our hits. The predicted protein structure of NALP5 includes a Pyrin domain followed by a nucleotide binding site (NBS) and a leucine-rich repeat. RT-PCR results showed that expression of NALP5 mRNA was induced in two models of neuronal injuries: serum/$K^+$ withdrawal in cerebellar granule neurons (CGN) and transient middle cerebral artery occlusion (MCAO) in rat. A potential function for NALP5 in apoptosis has not been described previously, and we sought to investigate this in recombinant systems. Transient expression of human NALP5 in cell lines (HeLa, HEK293, and 3T3) or in primary neurons (CGN) induced caspase-3 activation and apoptosis, as detected by DEVD-Fmk staining and Hoechst-33342 staining respectively. These data suggest that NALP5 may regulate programmed cell death in injured neurons via a caspase-3 dependent mechanism. This is the first time that NALP5 has been shown to have pro-apoptotic activities, and it is possible that this correlates with the induced gene NALP5 expression in injured neurons. NALP5 represents a novel molecular target for therapeutic intervention in neurodegenerative disorders.

1564

### PNPase Deficiency Compromises Apoptotic Signalling

S. W. French,[1] H. Chen,[1] R. N. Rainey,[2] D. W. Dawson,[1] C. E. Balatoni,[1] J. J. Troke,[1] J. Hong,[1] C. M. Koehler,[2] M. A. Teitell[1]; [1]Pathology, David Geffen School of Medicine at UCLA, Los Angeles, CA, [2]Chemistry and Biochemistry, UCLA, Los Angeles, CA

Mammalian polynucleotide phosphorylase (PNPase) is a 3' to 5' RNA exonuclease that is upregulated in terminally differentiated and senescent cells. However, overexpression of PNPase results in pleiotropic cellular effects that are difficult to reconcile from a presumed role in RNA turnover in the mitochondria matrix. Here, we show that PNPase is imported into mitochondria in the presence of a membrane potential and localizes to the intermembrane space (IMS) as a peripheral membrane protein. Functionally, truncated BID causes partial PNPase release from mitochondria following cytochrome *c* release, where it may encounter and degrade poly(A)+ RNA. PNPase knockdown causes increased cell survival and mitochondria membrane potential stabilization with growth factor withdrawal, indicating roles for PNPase in cell death and respiration. PNPase knockdown also inhibits ATP production and increases lactate levels in serum, further supporting a role for PNPase in oxidative phosphorylation. Combined, efficient cellular mechanisms in apoptosis and metabolism seem to require the novel, IMS-localized PNPase ribonuclease.

1565

### Inducing Endoplasmic Reticulum Stress in Chondrocytes Results in Loss of Differentiation, Cell Growth Arrest and Apoptosis

L. Yang,[1] S. G. Carlson,[2] D. McBurney,[1] W. E. Horton Jr.[1]; [1]Department of Anatomy, Northeastern Ohio Universities College of Medicine, Rootstown, OH, [2]The Lerner Research Institute of The Cleveland Clinic Foundation, Cleveland, OH

In eukaryotic cells, the Endoplasmic Reticulum (ER) is a major storage organelle for calcium and site of synthesis and folding of secretory proteins, cell membrane proteins, and is sensitive to changes in the internal and external environment of the cell. Both physiological and pathological conditions may perturb the function of the ER resulting in ER stress. The chondrocyte is the only resident cell found in cartilage and is responsible for synthesis and turnover of the abundant extracellular matrix and may be sensitive to ER stress. Here we report that glucose withdrawal, tunicamycin and thapsigargin induce ER stress in both primary chondrocytes and a chondrocyte cell line, detected by upregulation of GADD153 and caspase-12. Other agents such as IL-1β or TNFα induced a minimal or no induction of GADD153, respectively. ER stress resulted in decreased accumulation of an Alcian Blue positive matrix by chondrocytes and decreased expression of type II collagen at the protein level. Further, quantitative real time PCR was used to demonstrate a downregulation of steady state mRNA levels coding for aggrecan, collagen II, and link protein in chondrocytes exposed to ER stress-inducing conditions. The Sox9-dependent collagen type II promoter activity was downregulated under ER stress condition. In addition, the ER stress resulted in decreased chondrocyte growth based on cell proliferation assay, upregulation of p21 and decreased PCNA expression. Ultimately, ER stress resulted in chondrocyte apoptosis, as evidenced by DNA fragmentation and Annexin V staining. These findings have potentially important implications regarding consequences of ER stress in cartilage biology.

1566

### Involvement of Ataxia-telangiectasia-mutated (atm) in Calyculin A-induced Apoptosis

X. Tang, Z. Hui, B. Xu; Dept. of Genetics, Louisiana State University Health Science Center, New Orleans, LA

Calyculin A is a natural toxin produced by marine sponge and it is a specific and potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A). These phosphatases are essential for cellular signaling network and cell survival, they can also be explored as new targets for developing cancer therapeutic drugs. Exposing cells to calyculin A induces cell death, but the mechanism regulating calyculin A-induced cell death is not well defined. We report here that exposure to calyculin A activates cellular apoptotic response in a variety of cell lines and it is correlated with premature chromosome condensation and global histone H3 phosphorylation. Among the cell lines we tested, Ataxia Telangiectasia Mutated (ATM) -deficient cells displayed resistance to calyculin A-induced cell death detected by MTT assay and colony formation assay. Furthermore, ATM-deficient cells have a pronounced defect in calyculin A-induced apoptosis. These observations indicate that ATM is critical for cellular death induced by calyculin A. Further experiments revealed that ATM is activated in response to Calyculin A treatment and the Structural Maintenance of Chromosomes protein one (SMC1) is phosphorylated in an ATM-dependent manner. Over-expression of a mutated SMC1 (ATM-mediated phosphorylation site mutant) has a dominant negative effect that can abrogate the apoptotic response. These data suggest that ATM phosphorylation of SMC1 is the essential signaling pathway in initiating calyculin A induced apoptosis.

1567

**Programmed Cell Death of the Jacket Cells is Essential for Spermatid Development and Differentiation in *Marsilea vestita***

F. Deeb, S. M. Wolniak; Cell Biology and Molecular Genetics, University of Maryland, College Park, MD

The microspore of the water fern *Marsilea vestita* contains a single cell that is activated to undergo nine rapid division cycles after it is placed in water. The divisions produce seven somatic cells and 32 spermatids. The gametophyte is transcriptionally quiescent during its development and before the divisions commence, certain stored proteins and mRNAs localize in zones that later become the spermatogenous initials. Asymmetric divisions produce somatic jacket cells, and a series of symmetric divisions produce the spermatids. As the spermatids differentiate to become motile gametes, the jacket cells undergo programmed cell death (PCD). We developed RNAi strategies to target mRNA degradation and thereby arrest gametophyte development. These published studies show that the temporal and spatial translation pattern in the gametophyte is highly ordered; certain proteins are required at specific stages of development. In this study, we inhibited PCD in jacket cells with RNAi treatments. dsRNA probes were made from cDNAs encoding two different cell death associated proteins that we isolated from a gametophyte library. These dsRNAs were added to populations of spores and gametophytes were fixed after 8 hours of development. Treated spores had large jacket cells and fewer spermatogenous cells than control gametophytes. Toluidine Blue-O staining of treated spores showed that jacket cells contain more total proteins and mRNAs than the jacket cells in untreated spores. Treated cells were labeled with anti-centrin and anti-β-tubulin antibodies to assess patterns of new translation of stored mRNA and cytoskeletal reorganization, respectively. Unlike normal gametophytes, centrin translation and tubulin localization were no longer limited to the spermatogenous cells. We suspect that impending PCD may underlie the early suppression of translation and development in the jacket cells. (Supported by NSF grant MCB-0234423 to SMW).

1568

**The *S cerevisiae* Protein Bir1p Is a Substrate for Nma111p and Is Able to Inhibit Apoptosis in Yeast**

D. Walter,[1] S. Wissing,[2] F. Madeo,[3] B. Fahrenkrog[1]; [1]M.E. Mueller Institute for Structural Biology, Biozentrum, Basel, Switzerland, [2]Physiologisch-chemisches Institut, University of Tübingen, Tübingen, Germany, [3]Institute for Molecular Biosciences, University of Graz, Graz, Austria

Apoptosis is a form of programmed cell death that is crucial for the development and maintenance of multicellular organisms. Apoptosis is characterized by distinct morphological changes of the cell, and recent findings suggest that a form of programmed cell death also exist in unicellular organisms, such as the yeast *S. cerevisiae*. We have recently shown that the *S. cerevisiae* HtrA-like serine protease Nma111p is able to mediate apoptosis in yeast. In search for substrates of Nma111p that provide insight into the molecular mechanisms of Nma111p's proapoptotic activity, we have now identified Bir1p as a substrate for Nma111p. Bir1p belongs to the family of inhibitor-of-apoptosis proteins (IAPs) that are typically characterized by the presence of one to three baculovirus IAP repeat (BIR) domains that are essential for their anti-apoptotic activity. Bir1p is the solely BIR-protein in yeast and is known to participate in chromosome segregation events, whereas its putative role in yeast apoptosis has not been studied yet. We show here that yeast cells lacking *bir1* are more sensitive to oxidative stress induced by $H_2O_2$ than wild-type cells and undergo apoptosis more frequently. Consistently, overexpression of Bir1p reduces apoptotic-like cell death. This protective effect of Bir1p can be antagonized by simultaneously overexpressing Nma111p. Apoptosis can be induced physiologically by long-term cultivation, known as chronologically ageing. Disruption of *BIR1* significantly accelerates the onset of age-induced cell death, whereas the onset of cell death is delayed in yeast cells overexpressing Bir1p. These results indicate that Bir1p in fact is an inhibitor of apoptotic cell death in yeast, and, like its closest metazoan homologues deterin and survivin, seems to have dual functions: it participates in chromosome segregation and exhibits anti-apoptotic activity.

1569

**Par-4-mediated Recruitment of Amida to the Actin Cytoskeleton Leads to the Induction of Apoptosis**

M. Boosen,[1] S. Vetterkind,[1] A. Koplin,[1] S. Illenberger,[2] U. Preuss[1]; [1]Institute of Genetics, University of Bonn, Bonn, Germany, [2]Cell Biology, Zoological Institute, Technical University of Braunschweig, Braunschweig, Germany

Par-4 (prostate apoptosis response-4) sensitizes cells to apoptotic stimuli, but the exact mechanisms are still poorly understood. Using Par-4 as bait in a yeast two-hybrid screen, we identified Amida as a novel interaction partner, a ubiquitously expressed protein which has been suggested to be involved in apoptotic processes. Complex formation of Par-4 and Amida occurs *in vitro* and *in vivo* and is mediated via the C-termini of both proteins, involving the leucine zipper of Par-4. Expression analysis of Amida in rat fibroblasts revealed a predominantly nuclear localization. Upon coexpression with Par-4, Amida colocalized with Par-4 at actin filaments, resulting in enhanced induction of apoptosis in REF52.2 cells. A similar Par-4-mediated translocation to the microfilament system has previously been demonstrated for Dlk/ZIP kinase, a well characterized interaction partner of Par-4 (S. Vetterkind et al. Exp. Cell Res. 305, 392-408, 2005). Coexpression of Amida and the leucine-zipper point mutant Par-4 L3 resulted in abrogation of both Amida interaction and apoptosis. Furthermore, disruption of the microfilament system decreased apoptosis as well as coexpression of Amida and a Par-4 deletion construct that is impaired in actin binding, indicating that the Par-4-mediated relocation of Amida to the actin cytoskeleton is crucial for the pro-apoptotic function of Par-4/Amida complexes. We postulate that the recruitment of nuclear proteins involved in the regulation of apoptotic processes to the actin filament system by Par-4 represents a potent mechanism how Par-4 can trigger apoptosis.

1570

**Retinal Degeneration in Chronically Hypoglycemic Mice**

R. Barlow,[1] Y. Umino,[1] D. Everhart,[1] T. Nguyen,[1] J. Pan,[1] B. Frio,[1] R. Hafler,[1] B. Knox,[1] E. Solessio,[1] G. Engbretson,[1] K. Cusato,[2] M. Charron[3]; [1]Center for Vision Research, SUNY Upstate Medical University, Syracuse, NY, [2]Ophthalmology, UCSF School of Medicine, San Francisco, CA, [3]Biochemistry, Albert Einstein College of Chemistry, Bronx, NY

Our objective is to determine if metabolic stress in the form of hypoglycemia causes retinal degeneration. We examined the retinas of mice rendered chronically hypoglycemic by a null mutation the glucagon receptor gene, *Gcgr.* Homozygotes have about half normal blood glucose, and heterozygote and wildtype littermates have normal levels. We assessed retinal sensitivity (ERG), anatomy and optomotor responses of Gcgr+/+, Gcgr+/-, Gcgr-/- mice as a function of age. We found that retinas of Gcgr-/- mice begin to degenerate and the animals begin to lose visual acuity at

about 10 months of age and by 14 months the progressive changes are nearly complete, and the mice are functionally blind. Heterozygote littermates and wild-type mice retain normal visual acuity and normal retinal anatomy and sensitivity past 20 months of age. The molecular and cellular mechanisms underlying retinal degeneration are not known. TUNEL staining shows earliest cell death occurs in the inner nuclear layer followed by the outer nuclear and ganglion cell layers. The late-onset retinal degeneration in Gcgr-/- mice results from a null mutation of a gene that does not appear to be expressed in the retina. We suggest that the degeneration results from the metabolic stress caused by hypoglycemia resulting from the null mutation. The late-onset properties of retinal degeneration in these mice may make them useful models for studying causes of some human retinal diseases such as age-related macular degeneration. Support: NEI, NHLBI, NIDDK, NIMH, ADA, AECOM Comprehensive Cancer Center, Research to Prevent Blindness and Lions of Central NY.

1571

**Bcl-XL Enhances the Effect of GM-CSF in the in Vitro Development of the Monocyte/Macrophage Lineage**

A. Antignani, R. J. Youle; National Institutes of Health, Bethesda, MD

We have fused GM-CSF, via recombinant DNA, to Bcl-XL and expressed the recombinant fusion protein, GM-CSF-Bcl-XL, using bacteria and yeast. The goal of this chimeric protein is to bind the target human cells population through the GM-CSF receptor and prevent hematopoitetic cell loss during chemio or radiotherapy. Bcl-XL is a member of the Bcl-2 protein family and is able to suppress cell death induced by diverse stimuli in many cell types, including hematopoietic cells. Cytokine are the major mediators of host defense, in that they control hematopoietic cell proliferation, survival and regulate the communication between antigen-presenting cells (APC), lymphocytes and the other host cells. Human granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes the proliferation and maturation of neutrophils, eosinophilis, and macrophages from bone marrow progenitors. We found that the chimeric protein protects cells from staurosporine induced apoptosis from 24 hours until at least 72 hours, increasing the cell proliferation in monocyte culture. In presence of TyrAg490, an inhibitor of the Jak2 kinase, GM-CSF-Bcl-XL promotes proliferation whereas the cytokine alone is completely inhibited, because of the Bcl-XL portion. The protein is still active even in presence of specific anticancer drugs (cytarabine and daunorubicin). In all condition the antiapoptotic effect was measured and it is higher than the action of the GM-CSF alone. In presence of cytarabine and daunorubicin GM-CSF-Bcl-XL is able also to promote the differentiation of the CD34+ myeloid precursor, with an increase of the population of granulocytes compared to cells treated only with the fusion protein. We conclude that recombinant GM-CSF-Bcl-XL binds the GM-CSF receptor on human monocyte/macrophage cells and bone marrow progenitors and enters into the cells where Bcl-XL blocks cell death increasing the cell proliferation and differentiation.

1572

**Expression of Apoptosis Related Proteins in Villous Cytotrophoblast During Differentiation**

B. Wei, C. Xu, N. S. Rote; Reproductive Biology, Case Western Reserve University, Cleveland, OH

The human placenta is responsible for protection of the fetus during pregnancy. The placental villous surface is composed of 2 major types of cells, villous cytotrophoblast and syncytiotrophoblast. The mononuclear cytotrophoblasts form the inner layer of villi and differentiate to form the outer multinucleate syncytium, syncytiotrophoblast. Syncytiotrophoblasts are non-proliferate and rely solely on intercellular fusion with the underlying cytotrophoblasts for expansion during pregnancy. Although differentiating villous cytotrophoblasts undergo several changes resembling apoptosis, the role of apoptosis in villous cytotrophoblast differentiation is highly debated. To address this question, we studied the expression of a panel of apoptosis-related proteins during forskolin-induced differentiation of a model of villous cytotrophoblast, the choriocarcinoma cell line BeWo. Differentiating BeWo closely mimic differentiating villous cytrophoblasts (e.g., increased hCG production, decreased cellular proliferation, and intercellular fusion into syncytia). We observed that differentiation and syncytialization were not accompanied with activation of caspases-3, -8 or -9, yet the levels of pro-caspases were significantly diminished. By using real-time PCR, we showed that the decreased pro-caspases resulted from reduced transcription rather than activation as proposed by others. Moreover, expression of the pro-apoptotic proteins p53 and Bax were also down-regulated, while those of the anti-apoptotic protein Bcl-2 were up-regulated. Expression of Bcl-xl was unaffected. Expression of p53 protein was decreased within the first 24-hr of differentiation, and changes in Bcl-2, Bax, and the caspases protein levels were not significant until 48-hr of differentiation. Thus, differentiation of the villous cytotrophoblast model BeWo is concurrent with a shift in expression of apoptotic proteins towards a state of decreased apoptotic sensitivity. Whether this shift is related to the villous cytotrophoblast fusion process or whether it is a way of acquiring or maintaining an immune privileged status of the syncytiorophoblasts is under current investigation.

1573

**Wee1 Promotes Apoptosis during the Early Embryonic Development of *Xenopus laevis***

B. N. Wroble, J. C. Sible; Biology, Virginia Tech, Blacksburg, VA

In somatic cells, when endogenous DNA is damaged or unreplicated, checkpoint pathways function to arrest the cell cycle prior to M or S phases through the inhibition of cyclin-dependent kinases (Cdks). In *Xenopus laevis*, early embryonic cellular divisions 2-12 consist of rapid cleavage cycles in which gap phases, cell cycle checkpoint engagement, and an apoptotic program are absent. Upon the completion of the 12$^{th}$ cellular division, the midblastula transition (MBT) begins and the cell cycle lengthens, acquiring gap phases. In addition, cell cycle checkpoint pathways and an apoptotic program become functional. In *Xenopus laevis*, Cdks are inhibited by phosphorylation of tyrosine 15 by the Wee1 kinase. To determine the role of Wee1 in cell cycle checkpoints and the remodeling events of the MBT, exogenous wt-Wee1 was expressed in one-cell stage *Xenopus* embryos. Modest overexpression of wt-Wee1 created a pre-MBT cell cycle checkpoint, characterized by cell cycle delay and inhibitory phosphorylation of Cdks on tyrosine 15. Furthermore, overexpression disrupted key developmental events that normally occur at the MBT, such as the degradation of Cdc25A, cyclin E, and Wee1. Additionally, these embryos possessed a nucleo-cytoplasmic ratio below the critical concentration normally seen at the MBT. Interestingly, overexpression also resulted in post-MBT apoptosis, as determined by gross morphology and activation of caspases. Conversely, overexpression of wt-Chk1 and wt-Chk2, which normally creates a pre-MBT cell cycle checkpoint, inhibits apoptosis. Taken together, these data suggest the importance of Wee1 as not only a Cdk inhibitory kinase, but also as a promoter of apoptosis during the early development of *Xenopus laevis*.

1574

**Role of CK2 in Regulation of TRAIL-induced Apoptosis in Prostate Cancer Cells**

G. Wang, K. A. Ahmad, H. Lindvall, A. Johnson, G. M. Unger, J. W. Slaton, K. Ahmed; Research Service/Lab Med Path, V.A. Medical Ctr/Univ of Minnesota, Minneapolis, MN

Protein kinase CK2 (formerly casein kinase 2 or II) signal is involved in cell proliferation and also in suppression of cell death (apoptosis) (Ahmed et al., Trends Cell Biol 12: 226-230, 2002). Prostate cancer is one of the leading cause of death in the U.S.A. In an attempt to decipher the potential role of CK2 in receptor-mediated apoptosis, here we demonstrate that prostatic carcinoma cells, regardless of their phenotype (such as androgen-insensitive PC-3 or androgen-sensitive ALVA-41), are sensitized to TRAIL-mediated apoptosis by chemical inhibition of CK2 employing the specific inhibitor TBB (4,5,6,7-tetrabromobenzotriazole). Conversely, overexpression of CK2 by using pcDNA6-CK2α expression plasmid affords protection against TRAIL-induced apoptosis. The downstream targets impacted by CK2 in modulating the TRAIL functions are the death inducing signaling complex (caspase activity), DNA fragmentation, and cleavage of downstream target lamin A. Overexpression of CK2 also blocks the mitochondrial apoptosis machinery engaged initially by TRAIL. These findings suggest that while downregulation of CK2 sensitizes prostate cancer cells to TRAIL-mediated apoptosis, the overexpression of CK2 impedes the death receptor-mediated apoptotic execution thus providing insight into the mechanisms involved in CK2 mediated modulation of death receptor-mediated apoptosis. The results hint that novel anti-cancer strategies could be designed targeting CK2 by itself or in combination with TRAIL. [Supported by research grant from NCI and V.A. Medical Research funds].

1575

**Overexpression of Regucalcin Suppresses Apoptotic Cell Death in Cloned Normal Rat Kidney Proximal Tubular Epithelial NRK52E Cells: Change in Apoptosis-Related Gene Expression**

M. Yamaguchi, T. Nakagawa; Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, Shizuoka, Japan

The effect of regucalcin, a regulatory protein in intracellular signaling pathway, on cell death was investigated using the cloned normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin. NRK52E cells (wild type) and stable regucalcin (RC)/pCXN2 transfectants with subconfluent monolayers were cultured for 72 h in a medium without BS containing various factors. The number of wild-type cells was significantly decreased by culture for 72 h in the presence of TNF-alpha, LPS, Bay K 8644 or thapsigargin. These factor's effect was significantly prevented in transfectants. Agarose gel electrophoresis showed the presence of DNA fragments of adherent wild-type cells cultured with LPS, Bay K 8644 or thapsigargin for 24 h, and this DNA fragmentation was significantly suppressed in transfectants. TNF-alpha-induced decrease in the number of wild-type cells was significantly prevented by culture with caspase-3 inhibitor, while LPS- or Bay K 8644-induced decrease in cell number was significantly prevented by caspase-3 inhibitor or NAME, an inhibitor of NO synthase. Thapsigargin-induced decrease in cell number was not prevented in the presence of two inhibitors. Bcl-2 and Akt-1 mRNA levels were increased in transfectants, while Apaf-1, caspase-3 or G3PDH mRNA expressions were not changed in transfectants. Culture with TNF-alpha, LPS, Bay K 8644 or thapsigargin caused a significant increase in caspase-3 mRNA levels in wild-type cells. LPS significantly decreased Bcl-2 mRNA expression in the cells. Those effects on gene expression of apoptosis-related proteins were not changed in transfectants. This study demonstrates that overexpression of regucalcin has a suppressive effect on cell death and apoptosis induced by various factors which the action are mediated through many intracellular signaling pathways, and that regucalcin modulates the gene expression of apoptosis-related proteins.

1576

**Regulation of Bax Activation and Translocation by Mcl-1 During UV-induced Apoptosis in HeLa Cells**

K. Xiao, L. Zhou, D. C. Chang; Biology, HKUST, Hong Kong, Hong Kong Special Administrative Region of China

A key step to trigger the release of mitochondrial intermembrane proteins during UV-induced apoptosis is the Bax translocation from the cytosol to mitochondria to form small aggregates. The mechanisms for Bax translocation and aggregation, however, have not been fully understood yet. Apparently, other Bcl-2 family proteins play important roles in regulating Bax translocation and aggregation. Among them, Mcl-1 is one of the major regulators. We found that overexpression of Mcl-1 could efficiently prevent Bax translocation, suggesting that Mcl-1 can negatively regulate Bax activation during UV-induced apoptosis in HeLa cells. We observed that Mcl-1 degradation appears to play a significant role in Bax activation, since treatment of MG132, a proteasome inhibitor which prevents Mcl-1 degradation, could prevent Bax translocation. Despite of a transmembrane (TM) domain at its C-terminus, Mcl-1 was found to localize both in cytosol and mitochondria. We produced a cytosolic Mcl-1-ΔTM, in which the TM domain was deleted. We found that overexpression of cytosolic Mcl-1-ΔTM could significantly prevent UV-induced apoptosis, to the same extent as overexpressing full-length Mcl-1. However, Mcl-1-ΔTM could not prevent Bax overexpression-induced apoptosis, while full-length Mcl-1 could. Based on the above and other results, we propose a hypothesis that cytosolic Mcl-1 acts as the primary inhibitory step for Bax activation and translocation, while mitochondrial Mcl-1 prevents Bax forming aggregates at the mitochondrial outer membrane.

1577

**Bre Expression and Interdigital Apoptosis in the Developing Mouse Embryo**

W. M. Wong, K. K. Lee; Department of Anatomy, The Chinese University of Hong Kong, Hong Kong, Hong Kong Special Administrative Region of China

Brain and reproductive organ expressed (*Bre*) gene is a stress-responsive gene. It is highly expressed in brain, reproductive organs and adrenal gland. When HeLa cells are treated with UV-radiation, DNA-damaging agent or retinoic acid, BRE expression is down-regulated. The human *Bre* gene encodes a 1.8kb mRNA that translates into a 44kDa protein. The human Bre protein shares 99% homology in its peptide sequence with mouse and hamster. Bre has been previously shown to be associated with the juxtamembrane region of p55 tumor necrosis factor receptor-1 (TNFR1) and to down-regulate tumor necrosis factor (TNF) α -induced NFκB activation. It has also been shown to play an anti-apoptotic role in Hela and D122 cell lines. In our study, we have performed in situ hybridization and immunohistochemical staining to analyze the expression patterns of Bre, TNFR1 and NFκB in the mouse embryo in order to elucidate a role of Bre during development. These three genes and their protein products were found to co-express in the brain, heart, neural tube, and especially in the interverterbal and interdigital tissues which normally undergo apoptosis

during development. To further study the role of Bre and understand the relationship between Bre and apoptosis, embryonic limb cells were transfected with a Bre expression plasmid containing a GFP reporter. We demonstrated that over-expression of Bre induced apoptosis and did not alter the morphological appearance in the transfected cells. The differential expression of Bre in the mouse embryo, especially in apoptotic tissues, suggests that this protein may play a key role in the development, through modulation of TNF transduction pathway.

1578

**Anti-apoptotic and Pro-proliferative Effects of Basal Protein Kinase G (PKG) Activity in Vascular Smooth Muscle Cells (VSMCs), Determined Using the New Specific, Cell-permeable PKG Inhibitor DT-2**

C. Y. Wong, R. R. Fiscus; Dept. Physiol. (Fac. Med.) and Mol. Cell. Gerontol., Chinese University of Hong Kong, Shatin, NT, Hong Kong Special Administrative Region of China

Overstimulation of nitric oxide (NO)/cGMP/PKG pathway has pro-apoptotic effects in VSMCs, pancreatic beta-cells and colon cancer cells. Our data, however, indicate that NO at low physiological concentrations, via lower-level cGMP/PKG activation, (and even basal cGMP/PKG activity) has anti-apoptotic effects in many neural cells, female reproductive cells and pancreatic beta-cells. Therefore, NO has dichotomous regulatory roles in physiological and pathological conditions. (Over)stimulation of cGMP/PKG pathway is also reported to be anti-proliferative in VSMCs and vascular remodeling. Our present study determines the role of basal PKG activity, under normal culturing conditions, on apoptosis and proliferation in primary (P0) cultures of VSMCs (i.e. phenotypically normal VSMCs) using a newly-developed PKG inhibitor, DT-2. DT-2 is a highly specific (>10,000-fold selectivity for PKG compared to other protein kinases), potent membrane-permeant peptide blocker of PKG. Cell death detection ELISA was used to determine effects of DT-2 on apoptosis in primaryVSMCs from C57BL/6J mice (2-4 months). In presence of serum, DT-2 had no effect on apoptosis. However, without serum for 72 h DT-2 (250 nM) enhanced pro-apoptotic effect by 2-fold. Thus, basal PKG activity provided anti-apoptotic signal that limited apoptosis during serum deprivation. DT-2 (250 nM and 1 μM, 72 h) also significantly ($p<0.001$) decreased by 40% and 47%, respectively, the rate of cell proliferation stimulated by serum in primary VSMCs from C57BL/6J mice (2-4 months). These results suggested that PKG, at basal activity, plays an anti-apoptotic role and also enhances proliferation rate in primary, phenotypically-normal VSMCs. Support: RGC Competitive Earmarked Grant (CUHK4169/02M), a Direct Grant and a Strategic Grant to RRF.

1579

**New Players that Involved in Mouse Embryonic Interdigital Cell Death**

S. W. Shan, K. K. Lee; Department of Anatomy, The Chinese University of Hong Kong, Hong Kong, Hong Kong Special Administrative Region of China

Programmed cell death (PCD), or apoptosis, is an essential event in many kinds of biological processes and, the interdigital cell death is a typical example. PCD plays an important role in the shape of emerging limbs. The end result is the formation of free spaces that separate the digits in the hand and the foot. In this study we have used comparative proteomic techniques to identify proteins that may be involved in regulating the survival and death of mouse embryonic interdigital cells. We have identified many proteins that previously are not known to be expressed in the interdigital cells. Among these proteins are Protein Disulfide Isomerase (PDI) and Peroxiredoxin1 (Prdx1) which we found to be differentially expressed in interdigital tissues during PCD. For PDI, it was possible to inhibit interdigital cell death and expression of pro-apoptotic related genes (*bmp4* and *bambi*) by treating interdigital tissues with its enzyme inhibitor (bacitracin). These findings suggest that PDI is involved in the activation and maintenance of interdigital cell death. Conversely, when interdigital tissues were manipulated to survive Prdx1 was strongly expressed, but down-regulated when the cultures were allowed to die. Furthermore, we found that both *bmp4 and Dkk1* genes were down-regulated whereas the expression of another gene, *Wnt 5A*, was found maintained when interdigital tissues were manipulated to survive, these suggest that Wnt 5A may be one of the molecules that leads to the cartilage formation when the interdigital tissues were cultured. In sum, we have identify many new molecules involved in interdigital tissues and this implys that interdigital cell death is not a simple process but involves the interaction of proteins and genes that facilitate cell death and survival.

1580

**cIAP2-Associated Factor (C2AF): A Novel Protein that Controls Anti-apoptotic Function of cIAP2 and Promotes the Sumoylation of cIAP2**

H. S. Jin, H. S. Park, T. H. Lee; Yonsei University, Seoul, Republic of Korea

Cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2) are a family of proteins that suppress apoptosis induced by mitochondria-dependent and -independent pathways by inhibiting caspases. Structurally, the cIAPs contain three baculovirus IAP repeats (BIRs) which are responsible for the inhibition of caspase-3 and -7 as well as a C-terminal RING domain. The RING domains of cIAPs have been shown to possess E3 ubiquitin ligase activities, implying a connection between apoptosis regulation and ubiquitination-proteasome system. Here, we employed a targeted proteomic approach to screen proteins associated with cIAP2. After expression of flag-tagged cIAP2 in 293 cells, the endogenous proteins that are complexed with cIAP2 were purified and displayed on one-dimensional electrophoresis. Protein bands were processed for determining their identities by using mass spectrometic methods. Results reveal that in addition to several proteins previously known to interact with cIAP2, a novel protein was identified as a cIAP2-associated protein, which was named as C2AF. Expression of C2AF suppresses the anti-apoptotic effect of cIAP2 on TNF-induced apoptosis, and triggers a redistribution of cIAP2 from cytosol to the nucleus. We also found that both C2AF and cIAP2 are modified by SUMO conjugation, and C2AF promoted the sumoylation of cIAP2 in the nucleus.

1581

**The Growth Inhibition of MM Cells Induced by Agonist Anti-CD28 Monoclonal Antibody in vitro**

Q. Yu-hua, M. Hong-bing, S. Cheng-hai, S. Li-qin; Department of Immunology, Suzhou Medical College, Suzhou, China

CD28 expressed on T cells mediates necessary co-stimulatory signal promoting T cell activation, proliferation and function. CD28 expressed highly on human multiple myeloma (MM) cells but the significance hadn't been identified till now. Some researcher speculated that co-expression and engagement of CD28 and CD86 on MM cells might mediate a proliferation signals and consequently enhanced the proliferation ability for tumor cells. In this study, human multiple myeloma cell line U266, human T cell lymphoma cell line Jurkat and peripheral blood T cell (PBTC)

were chosen as targets. They were cultured in RPMI1640 medium with 10μg/ml agonist CD28 mAb. The results showed that the mAb had different effects on the different cell: (1) it inhibited growth and proliferation of U266 that highly expressed CD28 molecule and induced the cell apoptosis. Electrophoresis of DNA extract appeared a ladder band. Apoptosis bodies and chromatin marginal change were showed by transmission electron microscope (TEM); (2) it promoted proliferation of Jurkat and PBTC. The following explanation count for: (1) CD28 molecules expressed on different cells had a different configuration, possibly including isomers and mutants with different biological effects. (2) The expression density of CD28 was different on different cells. FCM analysis showed that the average fluorescence intensity of CD28 expressing on U266, Jurkat and PBTC was 462, 110 and 72, respectively. High cross-linking of antigens with the agonist CD28 mAb might inhibit cell membrane mobility, initiate and activate a certain inhibitory signals. The exact biological mechanism is still underlying and deserved to be investigated.

1582

**Visualization in Real Time of the Formation of the Mitochondrial Apoptosis-induced Channel, MAC, Triggered by T-bid by Patch-clamp Techniques**

S. Martinez-Caballero, L. M. Dejean, K. W. Kinnally; Basic Sciences, New York University CD, New York, NY

Bcl-2 family proteins tightly control apoptosis by regulating the permeabilization of the mitochondrial outer membrane and, hence, the release of pro-apoptotic factors from the intermembrane space. MAC is a high-conductance channel that forms early in apoptosis and is the putative cytochrome c release channel. Bcl-2 family proteins regulate apoptosis, in part, by controlling formation of MAC. Activated BH3-only proteins (e.g., t-Bid) trigger the release of cytochrome *c* in isolated mitochondria assayed by ELISA. Patch camp techniques were used directly on isolated mitochondria to verify that t-Bid can catalyze the formation of the MAC channel. The current flow across the outer membrane increased with time after seal formation, consistent with induction of MAC activity, when t-Bid was included in the micropipette. This MAC activity was reversibly blocked by dibucaine. Full length Bid or vehicle alone did not induce MAC formation nor release cytochrome c from isolated mitochondria. Various cell lines that do or do not express the multi-domain pro-apoptotic Bax and Bak are presently being examined. Our preliminary findings support the idea that t-Bid can regulate the formation of MAC in the mitochondrial outer membrane early in apoptosis. Supported by NIH grant GM57249 and NSF grant MCB-0235834 to KWK.

1583

**RNAi-Mediated Gene Silencing to Examine the Biological Function of a Tumor-Associated NADH Oxidase (tNOX) in Transformed Cells in Culture**

S. C. Liu, S. Huang, P. J. Chueh; Institute of Biomedical Sciences, National Chung Hsing University, Taichung, Taiwan Republic of China

Unbalanced cell survival and cell death can result in the development of cancer. In many cancer cells, the survival proteins are up-regulated whereas the pro-apoptotic proteins are down-regulated. Therefore, recent interests have been focused on identifying the anti-survival pathways for the development of cancer therapy. Previous reports have described a tumor-associated NADH oxidase (tNOX) and its constitutive activation in transformed culture cells. Certain anticancer drugs have been shown to inhibit preferentially the tNOX activity and growth of transformed culture cells that correlate with the induction of apoptosis. Here, we utilize RNA interference (RNAi) approach to investigate the biological function of tNOX in transformed cells and its association in apoptosis induction. In this study, the expression of tNOX protein is reduced in RNAi-transfected cells when compared with wild type HeLa cells. The reduction of protein expression is correlated with the decline of growth of RNAi-transfected HeLa cells. To further investigate the effect of tNOX gene silencing, we examine the key regulators of cell cycle by Western blot analyses. In addition, the apoptosis induction is enhanced in tNOX-RNAi cells as shown with flow cytometry using FITC-Annexin V/PI staining. Moreover, the mechanism of apoptosis induction by anticancer drugs is studied. In summery, our data have shown the involvement of tNOX in cell cycle and apoptosis by utilizing RNAi-mediated gene silencing and these fundamental understanding of biological role of tNOX may lead to new anti-cancer strategies. (Supported by NSC93-2311-B-040-001 and NHRI-EX94-9411BC)

1585

**A Role of Zyxin in Apoptosis**

M. Hervy, M. C. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Zyxin is an evolutionarily conserved protein that is concentrated at sites of cell adhesion and along actin stress fibers. The protein interacts with a variety of partners and is postulated to serve as a docking site for the assembly of multimeric protein complexes involved in regulating cell motility. Zyxin is also known to shuttle between the cytoplasm and the nucleus. Furthermore *zyxin* gene expression has been reported to be misregulated in a variety of tumors. In an effort to explore zyxin functions that might be relevant in tumorigenesis, we have investigated the involvement of zyxin in apoptosis, a process that is often disturbed in tumor cells. In particular we have compared the effect of UV-C irradiation in wild-type and zyxin-null mouse fibroblasts. We found that, after UV-C treatment, zyxin-null cells had an increased survival rate correlated with slightly lower caspase-3 activation. This observation led us to hypothesize that this effect could be mediated by an interaction of zyxin with regulators of apoptosis. We thus engineered a murine zyxin-null cell line which expressed a tagged version of zyxin allowing for the tandem affinity purification of protein complexes. This approach led us to recover the protein CCAR1 (Cell Cycle and Apoptosis Regulator 1) and further analysis allowed us to identify CCAR1 as a direct partner of zyxin. CCAR1 has recently been described in human breast cancer cells as a mediator of the apoptosis induced by the retinoid CD437, but little is known concerning the function and the mode of action of this protein. The functional significance of the interaction of these two proteins is currently under investigation and could point to a role for zyxin in the regulation of apoptosis.

1586

**RhoB Expression in Kidney Tubules in Response to Ischemia**

M. A. Hallett, N. Ray, S. J. Atkinson; Medicine, Indiana University, Indianapolis, IN

RhoB has been shown to be important for the response to various cellular stresses and is the target of farnesyl transferase inhibitors that induce apoptosis. RhoB may function by antagonizing the Akt survival pathway. We were interested to know if RhoB might be important for apoptosis in kidney tubules that occurs following ischemia. To determine if RhoB expression is changed by ischemia, in situ RTPCR was used to probe rat

kidney sections at 3 and 24 hours after 30 minutes of renal pedicle occlusion. We found that at 24 but not 3 hours post ischemia, RhoB mRNA was elevated. To determine if RhoB protein expression is also increased after ischemia, mouse kidney was made ischemic for 32 minutes, removed quickly, snap frozen in liquid nitrogen and pulverized. A detergent extract was used for a RhoB pull-down assay. We found that RhoB protein expression was elevated at 3 and 24 hours compared to the sham operated control. More of RhoB was active at 3 hours post ischemia than at 24 hours, suggesting that the stabilization of RhoB protein may occur before the increase in RhoB message. The interaction of RhoB with the Akt signaling pathway is our current focus. We suggest that the regulation of RhoB expression may be an important response to kidney ischemia.

1587

**A Mechanochemical Model Linking One-Hit Apoptosis to Cytoskeletal Dynamics**

T. Lomasko, C. J. Lumsden; Medicine, University of Toronto, Toronto, ON, Canada

The constant risk of cell death observed in neurodegenerative diseases may result from a “one-hit mechanism” (OHM) in which a catastrophic event triggers apoptosis (*Nature*,**406**,195). However, the role of the cytoskeleton (CSK) in the OHM has not been thoroughly explored. We have described an idealized mathematical model in which OHM biochemical subunits were distributed along a viscoelastic CSK filament. Filament shortening brings the subunits together and induces apoptosis. Our previous model dealt with a simplified kinetic scheme in which the rate at which stochastic changes in filament length take place is inversely proportional to the size of the changes (*Mol.Biol.Cell*,**15**,135a:748), i.e. the filament fluctuation kinetics resembles a well stirred chemical reaction. Here we treat a more realistic kinetics in which the rates and magnitudes are not locked together. We report simulation results for individual cells and 1-dimensional cell populations when one universal kinetic rate characterizes filament length changes of all sizes. A self-consistent hybrid scheme simulated filament length change as a stochastic variable and the kinetic state of downstream death trigger and intermediate messengers as deterministic nonlinear variables. We determined that for a single model cell, the spontaneous death rate is positively correlated with the magnitude of the stochastic deformations. In our computer models of the cell populations, we observed traveling waves and patches of cell death similar to those reported for diseased retinas (*Bull.Math.Biol.*,**64**,1117). Deletion of apoptotic cells from the population increased the mechanical stress impinging on all remaining cells, further enhancing the feedback from tissue mechanics to death kinetics. Our method is readily generalized to cell populations distributed in two and three dimensions. We conclude that our model simulates key experimentally observed cell death patterns, and therefore help clarify the role of the CSK in degenerative diseases.

1588

**Nongenomic Pathway for Anti-Estrogenic Signaling by Cadmium in Estrogen-Induced Osteoclast Apoptosis**

J. Rink,[1] A. K. Wilson[2]; [1]Northwestern University, Chicago, IL, [2]Biological Sciences, Benedictine University, Lisle, IL

Low-level cadmium exposure has been demonstrated to induce bone loss *in vitro* and *in vivo* by increasing the bone resorbing activity of osteoclasts. Osteoclasts normally undergo apoptosis shortly after they are activated. However, low-level cadmium exposure delays the onset of apoptosis at least 24 hours allowing the osteoclast more time to resorb bone. We investigated the signaling pathways for delaying estrogen-induced apoptosis. Apoptosis was induced in differentiated osteoclasts by the addition of 1μM β-estradiol in the presence or absence of 100 nM cadmium for 24 h. The percentage of apoptotic cells present was determined by morphometric analysis using fluorescent microscopy. β-estradiol and PPT, an estrogen receptor-α agonist, both increased the percent apoptosis approximately two-fold above baseline values. Coincubation with cadmium brought apoptosis values back down to the baseline level. Coincubation with the estrogen receptor antagonist, ICI 182780 at concentrations that diminished estrogen-induced apoptosis approximately 50%, could be further rescued by cadmium to the baseline level suggesting that cadmium is acting through the estrogen receptor. Similar results were obtained after inducing apoptosis with estren, an estrogen agonist that works exclusively in a nongenomic manner. Preincubation with the MEK inhibitor, PD98059 or the PI3K inhibitor, wortmannin did not inhibit estrogen-induced apoptosis. Cadmium was unable to inhibit estrogen-induced apoptosis after preincubation in PD98059 whereas it was able to completely prevent estrogen-induced apoptosis after preincubation in wortmannin. These data suggest that cadmium is interacting with the cell surface estrogen receptor-α but may be using an alternate pathway for cell survival other than MEK/ERK. Supported by HHMI grant #52002669 and the Scholl Foundation.

1589

**Counteracting Effect of Boron on the Structural Changes in Cytochrome C Exposed to Peroxides**

H. Farooqi,[1] S. Ali,[1] F. Ahmad,[2] M. Baig[1]; [1]Biochemistry, Hamdard University, New Delhi, India, [2]Biosciences, Jamia Millia Islamia, New Delhi-110025, India

Mitochondrial cytochrome c is a globular 12 KDa heme protein that is also recognized as an apoptotic inducer protein. Reactive oxygen species such as peroxides, formed *in vivo*, have been reported to inflict damage to this protein, and ultimately cause its release from the mitochondria into cytosol. Cytosolic cytochrome c then triggers various signal transduction pathways culminating in cell death. In the present study, we have shown that under oxidative stress conditions *in vitro* cytochrome c is damaged. The damage has been studied by spectrophotometric, gel filtration and electrophoretic techniques. Boron can counteract this damage as is evident from the results obtained using the above mentioned techniques. This suggests that boron stabilizes the protein structure. This stabilization may be due to interaction of tetrahedral borate anion. $B(OH)^-_4$ , which structurally resembles phosphate ion. Earlier NMR studies have shown that cytochrome c has at least two binding sites for borate. The study has implications in understanding the mechanisims of cell death.

## Mitosis & Meiosis III (1590-1618)

1590

**Isolation and Mapping of Mini-Chromosome-Loss Mutations in Caenorhabditis elegans**

M. Olaf, R. Tooley, S. Keller, M. Howe; Department of Biology, Bucknell University, Lewisburg, PA

Chromosome capture, and movement during mitosis are processes essential to chromosome segregation, and each requires kinetochore-spindle interactions. We have developed a sensitized genetic screen to identify proteins that mediate kinetochore-spindle interactions. In *C. elegans* chromosomes are holocentric allowing extrachromosomal transgenes to segregate as mini-chromosomes during mitosis. Chromosomal fragments

and transgenes regularly segregate as mini-chromosomes at mitosis and meiosis, because they, too, assemble kinetochores. We have shown that transmission of these mini-chromosomes is more sensitive to a mutant form of the kinetochore protein, HIM-10, than are whole chromosomes. Using free duplications or transgenes, marked with a GFP reporter construct, we have screened for mutations, like *him-10*, that cause mini-chromosome loss. Specifically, we screened for mosaic expression of the GFP reporter using the *him-10* mutant as a positive control. Six candidate mutations were isolated that cause mosaic GFP expression. Secondary analysis of these mutations involves determining that GFP expression from the duplication and transgene is mosaic and transmission of mini chromosomes is reduced in the germ line. We are developing assays to directly visualize the mini chromosomes in embryonic and germ-line cells. One mutation, *qa5300*, exhibits high frequency loss of the transgene. Transmission of the transgene through the germ line in this mutant was reduced by 18-20% when compared with the parent strain and similar to transmission in a *him-10* control strain. Similarly, DAPI staining of the germ line suggested that the frequency of transgene in diakinesis oocytes was reduced by 18-20% in the *qa5300* worms when compared to the parent strain. The mutation was mapped to the third chromosome using traditional methods. The map position of *qa5300* is being refined using SNP markers. In summary our result suggest that mini-chromosome loss is an effective primary assay for mutations affecting mitotic segregation.

1591

**Characterization and Mapping of Mini-Chromosome-Loss Mutant *qa5301* in *C elegans***

S. Eggert, M. Howe; Bucknell University, Lewisburg, PA

The overall aim of our research is to understand the molecular machines that segregate chromosomes at mitosis. Specifically we are identifying proteins that mediate kinetochore-spindle interactions using a sensitized genetic screen for mutations that cause mini-chromosome loss in *C. elegans*. We have identified a candidate mutation, *qa5301*, that causes mosaic expression of a GFP reporter construct carried on a transgenic mini chromosome. The mosaic phenotype of *qa5301* is similar to the phenotype observed in our positive control, a mutant version of the conserved kinetochore protein HIM-10. The mini chromosome is more sensitive to mitotic mutations than the intact chromosomes, allowing us to isolate homozygous viable mutant alleles such as *qa5301*. DAPI staining of diakinesis oocytes suggest that *qa5301* causes a low level of nondisjunction of the mini-chromosome sister chromatids in germ-line mitosis. There was a 10-fold increase in the number of oocytes with two mini-chromosomes in the mutant relative to the parental strain. Finally, though *qa5301* was not isolated as a temperature-sensitive mutation, it causes some sterility and larval arrest at elevated temperatures. These phenotypes are associated with other mitotic mutants in *C. elegans*, and they are consistent with chromosome segregation defects. Current efforts are focused on mapping the *qa5301* allele using a single nucleotide polymorphism technique with two reproductively isolated strains of *C. elegans*. The results thus far indicate that the *qa5301* allele is strongly linked to a marker on the right end of the X chromosome. Our results suggest that mapping and characterization of the gene impaired by the *qa5301* mutation will identify a factor important to proper mitotic chromosome segregation.

1592

**Chk2 Function During DNA Damage Induced Mitotic Catastrophe**

H. Varmark,[1] J. Nordberg,[2] W. Theurkauf[1]; [1]Molecular Medicine, University of Massachusetts, Worcester, MA, [2]Cell Biology, University of Massachusetts, Worcester, MA

The G2/M damage checkpoint delays progression into mitosis to allow time for repair, and thus helps prevent mitotic propagation of potentially deleterious mutations. When checkpoint control fails and cells enter mitosis with DNA lesions, mitosis is aborted through a poorly understood process termed mitotic catastrophe. It is unclear if division failure is a non-specific consequence of damage or the result of a genetically programmed damage response. We have previously shown that the Chk2 kinase is required for damage induced division failure in *Drosophila* embryos (Takada et al. Cell 2003). Chk2 transduces DNA damage signals to downstream targets that regulate cell cycle progression, DNA repair and apoptosis, and chk2 mutations in humans are linked to cancer predisposition. The mitotic functions for human Chk2, however, are not well understood. We have used chromosomal mutations in chk2 and several other checkpoint genes in human HCT116 colorectal cancer cells to examine the function of this conserved kinase during mitosis. Using FACS, live cell imaging, and immunofluorescence, we examined cell cycle progression and mitosis in cells treated with DNA damaging drugs. In the presence of DNA damage, a fraction of parental and checkpoint compromised p21-/- HCT116 cells progress into mitosis, delay in M-phase, and then exit mitosis without cytokinesis. During mitotic delay, spindles are abnormal and chromosomes are not properly aligned at the metaphase plate. By contrast, chk2-/- cells treated with DNA damaging agents do not delay in mitosis. Rather, anaphase figures are observed and cytokinesis is often completed. These observations indicate that human Chk2 is required to delay mitotic exit and block cytokinesis in response to DNA damage. One of the structures disrupted during this damage response is the mitotic spindle, suggesting that Chk2 links cytoskeletal organization to genome integrity during the mammalian mitotic catastrophe response.

1593

**Role of DNA Repair Proteins Involved in the Formation of Anaphase Bridges**

C. Acilan, W. S. Saunders; Biological Sciences, University of Pittsburgh, Pittsburgh, PA

Anaphase bridges, a commonly observed segregation defect in cancer cells, is one of the major sources of genomic instability. Bridges can theoretically be produced by any of several mechanisms including telomere-telomere fusion, persistence of chromatid cohesion into anaphase or repair of broken DNA ends. DNA damage can induce anaphase bridges following exposure to agents such as hydrogen peroxide or ionizing radiation. To determine how and why cells form bridges, we first set out to determine what DNA repair pathways the cells use to heal the damage and form bridges. Our data suggest that neither of the two major pathways, used by the cell for repair of double strand breaks, homologous recombination (HR) and non-homologous end joining (NHEJ), alone, is required for bridge formation. In fact, the NHEJ pathway seems to play a role in the prevention of bridges. When NHEJ is defective, cells appear to use HR to repair the DNA resulting in a relatively high frequency of bridges. Next, we surveyed whether we can explain the bridge forming potential in cancer cells with their NHEJ activity or the level of repair proteins. While the level of NHEJ proteins Ku80 and Ku70 does not show a relationship, it appears that XRCC4 levels can be used to estimate the tendency to form bridges. Our data also indicate that bridge frequency has a positive correlation with intrinsic NHEJ activity as judged by an in vitro ligation assay.

1594

**Life after Cleavage Failure: Centrosome Amplification and Cell Viability**

A. M. Krzywicka-Racka, G. Sluder; Cell Biology, University of Massachusetts Medical School, Worcester, MA

Persistent centrosome amplification (more than two centrosome at mitosis) is believed to cause aneuploidy, genomic instability, and promote the evolution of the transformed state. Cleavage failure clearly leads to immediate centrosome amplification and is regarded as a primary source for persistent centrosome amplification. We test whether cleavage failure per se can provoke persistent centrosome amplification in normal, immortalized human cells. Asynchronous hTERT-RPE1 cells are treated for 4-11 hours with cytochalisin D to induce cleavage failure resulting in ~30% incidence of binucleate cells. This regime is repeated another 4 times at 5 day intervals; at each round we passage a portion of the culture 7 times. We monitor the incidence of centrosome amplification before each round of cleavage failure, four days thereafter, and at the end of 7 passages. Despite repeated rounds of massive cleavage failure, we do not find the generation of a population of cells with persistent centrosome amplification. Extended time lapse observations of binucleate cells revealed that ~80% enter mitosis and usually divide in bipolar fashion due to spindle pole bundling. However, progressively fewer progeny enter subsequent mitoses; they become quiescent and do not die. None of observed binucleate cells divide more than 4 times whilst same preparation mononucleate cells continue dividing at >95% frequency. We conclude that cleavage failure leads to a reduction in viability and is insufficient to induce persistent centrosome amplification. Cleavage failure, however, may be a problem for cells that have other regulatory defects.

1595

**Does Aneuploidy Drive Tumorigenesis?**

B. A. A. Weaver, A. D. Silk, F. R. Putkey, D. W. Cleveland; Cell Biology, Ludwig Institute for Cancer Research, La Jolla, CA

For 100 years aneuploidy has been recognized as a frequent characteristic of cancer cells and has been hypothesized to cause tumorigenesis. To test this, we have created mice that produce elevated rates of aneuploidy from reduction in CENtromere associated Protein-E (CENP-E). CENP-E is an essential, kinesin-like motor protein that is accumulated and degraded in a cyclin-like pattern, localizes to kinetochores, and promotes stable kinetochore-microtubule interactions. Mitotic cells lacking CENP-E fail to attach and align a portion of their chromosomes, resulting in an elevated rate of missegregation (25% of divisions in vitro, 95% of hepatocyte divisions in vivo). CENP-E heterozygous mice are viable and fertile, but fibroblasts (MEFs) derived from CENP-E heterozygous mice are shown to exhibit 33% aneuploidy after 6 divisions in culture and 56% aneuploidy after 12 divisions. Lymphocytes from peripheral blood in CENP-E heterozygous mice were also determined to exhibit a high degree of aneuploidy (34% in 3 month old animals, rising to 66% by 10 months). Four in vivo experimental tests have been used to test the aneuploidy hypothesis. First, CENP-E heterozygous MEFs immortalized with SV40T antigen were determined to form foci in soft agar and have been injected into nude mice to test for the ability to form tumors. Second, the propensity of CENP-E heterozygous mice for development of tumors has been determined. Abnormal growths have been found in 29% of ovarian and 11% of spleen and lung tissues from aged (18-20 months old) mice heterozygous for CENP-E. Third, mice have been treated with chemical carcinogen (DMBA) to test the effect of elevated aneuploidy on tumor initiation. Fourth, cohorts of CENP-E heterozygotes that also lack the p19 tumor suppressor gene have been produced. Together, these approaches provide a direct test of the potential causal link between aneuploidy, tumorigenesis, and tumor progression.

1596

**Metabolism of Tasidotin (ILX-651) by Cancer Cells: Mechanisms of Activation and Resistance**

R. Bai,[1] M. C. Edler,[1] T. D. Copeland,[2] S. Schmid,[3] L. E. Arthaud,[3] E. Hamel[1]; [1]Pharmacology and Toxicology Branch, National Cancer Institute at Frederick, Frederick, MD, [2]Laboratory of Protein Dynamics and Signaling, National Cancer Institute at Frederick, Frederick, MD, [3]Genzyme Oncology, Genzyme Corporation, San Antonio, TX

Intracellular metabolism of tasidotin, an analog of the marine depsipeptide dolastatin 15, is the *t*-butyl amide of *N*,*N*-dimethyl-Val-Val-*N*-methyl-Val-Pro-Pro (the "metabolite"). Tritiated tasidotin, with only the fifth residue (the second proline) radiolabeled, was initially examined in Burkitt lymphoma CA46 cells to measure uptake and potential metabolism. Three prominent intracellular radiolabeled HPLC peaks were observed at all time points (5 min to 96 h). The earliest eluting peak, identified as proline, fluctuated between 25-40% of radiolabel recovered. The second peak, identified as the metabolite, steadily rose with time, from 18 to 60% of recovered radiolabel. The last peak, identified as tasidotin, steadily fell with time, from 49 to 1% of recovered radiolabel. Since the metabolite is a more potent inhibitor of tubulin than tasidotin or dolastatin 15, and earlier studies had indicated that removal of the terminal proline from the metabolite produced an inactive compound, these findings suggested that the Burkitt cells not only activated tasidotin but also could partially detoxify the compound. NCI drug screening data suggested that T47D breast cancer cells, and three other cell lines, should be resistant to tasidotin. At 5 h, 90% of the radiolabel recovered from the breast line was as proline, and at 24 h proline was the only radiolabeled peak. In the other three cell lines the proline content at 5 and 24 h ranged, respectively, from 50-70% and 50-95%. We plan to examine the NCI breast cell lines that should be sensitive to tasidotin for differences from the T47D line and the Burkitt line.

1597

**Nanoparticles Cause More DNA Damage in Human Cells Than Microparticles**

I. Papageorgiou,[1] E. Ingham,[2] R. P. Schins,[3] C. P. Case[1]; [1]Dpt. of Orthopaedic Surgery, University of Bristol, Bristol, United Kingdom, [2]Dpt. of Medical Immunology,School of Biochemistry and Microbiology, University of Leeds, University of Leeds, United Kingdom, [3]Particle Research, Institut fur Umweltmedizinische Forschung, Heinrich-Heine University, Dusseldorf,, Germany

There is currently much interest in the application of nanotechnology to medicine. In parallel with this, however, there is a concern about an adverse effect of exposure to nanoparticles, particularly if they are present in ambient pollution. In this study we have compared the biological effects of exposure of primary human fibroblasts to nanoparticles (29.5+/-6.3 nm) and microparticles (2.9±1.1μm) of cobalt chrome alloy. The release of cytokines was assayed by ELISA and cell viability by MTT and LDH assays. The amount of DNA and chromosome damage was quantitatively analysed by the Alkaline Single Cell Gel Electrophoresis (COMET) assay with image analysis and the micronucleus Assay with FISH-labelling with pancentromeric probes. Free radical production was measured by EPR spin trap. CoCr particles caused DNA damage in a dose-dependent way. However, small particles caused more DNA damage and at lower volumetric concentrations (0.005 $\mu m^3$/cell) than larger

particles (50 $\mu m^3$/cell). The difference in the threshold point was related to the surface area of the particles. With regard to the possible mechanism of the damage, smaller particles induced more superoxide and hydroxyl free radicals than larger particles. The DNA damage was correlated with a delayed decrease in cell viability, after three days of exposure. Both sizes of CoCr particles induced micronuclei in a dose dependent manner. No significant difference was noted but the nanoparticles caused significantly more centomeric-positive micronuclei than the microparticles. No significant amounts of cytokines (IL-6, TNF-a, TGF-beta-2, IL-10) were released from the fibroblasts after exposure to either particle. However the growth factor FGF-23 was increased in a similar pattern to the DNA damage. This study has demonstrated significant differences in the genotoxic,cytotoxic and mutagenic responses of human cells to nanoparticles and microparticles.

1598

**Evaluation of Toxicity of Ceramic Particles Within Human Cells *in vitro***

A. D. Tsaousi,[1] S. S. Taylor,[2] E. Jones,[1] C. P. Case[1]; [1]Dept. of Orthopaedic Surgery, University of Bristol, Bristol, United Kingdom, [2]School of Biological Science, University of Manchester, Manchester, United Kingdom

Problems arising from wear particles of the routinely used Metal-On-Polyethylene articulation in Total Hip Replacement (THR) surgery led to the development of novel materials. Ceramic-On-Ceramic articulation produces much less wear than any other combination. The greater need for implants in younger, more active patients necessitates research on the long-term effects of THR. This project has investigated the biological effects of alumina ceramic particles, a novel implant material relevant to orthopaedic surgery. Primary human fibroblasts were treated (in culture) with a variety of doses of alumina ceramic nanoparticles (ranging from 6.25 to 1000 ug/mL) for up to 5 days. The Trypan Blue Exclusion showed no significant differences in cell viability between control and treated cells, in all doses studied, up to 5 days of treatment. Subsequent microscopy analysis (after 24h of treatment with 25ug/mL) confirmed the existence of nanoparticles within cells. Beta-tubulin staining (immunofluorescence) did not reveal any major morphological differences in the cytoplasm of treated cells (with doses up to 250 ug/mL) when compared to controls. The Micronucleus Cytochalasin-B Genotoxicity Assay showed an increase in micronuclei formation after treatment with alumina nanoparticles (up to 0.2% versus 0.05% in controls). Interestingly, despite the wide range of doses tested (0-250ug/mL), there was no clear dose response. When combined with DNA pan-centromeric Fluorescent In-Situ Hybridisation, the above assay demonstrates that the level of aneugenic damage (measured as percentage of centromere positive micronuclei) is relatively high after ceramic treatment (40% versus only 12.5% in controls). Using the Anaphase-Telophase assay, anaphase bridges and lagging chromosomes were clearly seen in almost 9% of the treated cells (50ug/mL for 24h) while control values were less than 4%.In summary, the above suggests that alumina ceramic nanoparticles may cause aneuploidy in human cells.

1599

**Incenp Dynamics and Function in Vertebrate Mitosis**

L. J. Ahonen,[1,2] U. Klein,[3] M. Bolton,[4] E. A. Nigg,[3] T. Stukenberg,[4] M. J. Kallio[1]; [1]Cancer Biology and Cell Screening, VTT Medical Biotechnology, Turku, Finland, [2]TuBS Turku Graduate School of Biomedical Sciences, University of Turku, Turku, Finland, [3]Department of Cell Biology, Max-Planck-Institute of Biochemistry, Martinsried, Germany, [4]Department of Biochemistry and Molecular Genetics, University of Virginia Medical School, Charlottesville, VA

Inner centromere protein (Incenp) is a “passenger protein” essential for the ordered execution of mitosis. Incenp exhibits a transient localization pattern during cell division: first it accumulates to chromosome arms and inner centromeres at prophase, then translocates to the microtubules of the central spindle at anaphase, and finally concentrates to the midbody at telophase. Incenp is an integral component of the Aurora B complex that regulates microtubule-chromosome associations, spindle checkpoint signalling, and cytokinesis. Here we have measured the turnover of Incenp at the inner centromeres and spindle microtubules by using Fluorescence Recovery after Photobleaching (FRAP). The recovery of GFP-Incenp was recorded at different phases of normal cell division as well as in the presence of various drugs that either induce spindle damage or inhibit Aurora B kinase activity. The preliminary experiments show that Incenp is very mobile during early phases of mitosis but stabilizes at the onset of anaphase when the protein moves from inner centromeres to central spindle microtubules. In another approach, the normal function of Incenp was neutralized by anti-Incenp antibodies microinjected into mitotic Xenopus tissue culture cells. Cells injected at early mitosis were unable to align their chromosomes and exited mitosis prematurely without anaphase and cytokinesis. Similarly, cells with spindle damage and hyperactive spindle checkpoint rapidly decondensed their chromosomes, exited M phase without cell cleavage, and formed polyploidy progeny cells after introduction of anti-Incenp antibodies. These results indicate that Incenp is required for normal chromosome alignment, cell cycle arrest in response to spindle and chromosome errors, and execution of cytokinesis. Our results underline the importance of Incenp and Aurora B complex in promotion of bi-polar chromosome orientation and maintenance of spindle checkpoint activity, and identify Incenp as a potential target for future cancer therapies.

1600

**Characterization of Alternatively-Spliced Isoforms of INCENP**

W. J. Moore, E. Enervald, J. R. Swedlow; School of Life Sciences, University of Dundee, Dundee, United Kingdom

Aurora-B, a mitotic kinase involved in chromosome condensation, chromosome segregation and cytokinesis, is overexpressed in a number of tumor types. To investigate how the activity of Aurora-B changes in tumors, we sought to identify mutant alleles of the kinase and its regulators INCENP and Survivin in mRNA samples from a range of tumors. Sequence analysis revealed several splice variants of INCENP, a critical binding partner and regulator of Aurora-B. These consisted of alternatively spliced short (12bp) exons at two sites in the coding sequence of INCENP, encoding an insertion of the residues CSFV between exons 9 and 10 and VRAQ at the end of exon 12. Alternative splicing at these two sites appears to be independent. Quantitative PCR (qPCR) analysis showed that relative amounts of the CSFV insert fluctuated between tumor and normal samples and between different normal tissues. The VRAQ insert comprises ~23% of total INCENP across all normal tissues tested, but was elevated in some tumors. An anti-CSFV peptide antibody specifically recognized a biochemically distinct complex in HeLa lysates fractionated on a Superose 6 column. Splice variant specific siRNAi abolished expression of INCENP splice variants but resulted in only mild phenotypes characteristic of reduced INCENP activity, suggesting redundancy of function. We are currently defining the association of different INCENP splice variants with centromeres using FRAP and biochemically defining their components.

1601

**The Fission Yeast Aurora Kinase, Ark1, Promotes Bipolar Chromosome Segregation in Mitosis and Meiosis**

S. Hauf,[1] T. S. Kitajima,[2] Y. Watanabe[2]; [1]Friedrich-Miescher-Laboratory of the Max-Planck-Society, Tuebingen, Germany, [2]Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan

Work in a number of organisms has indicated that members of the Aurora kinase family are required for the correct bipolar segregation of sister chromatids in anaphase. In particular, Aurora kinases halt the cell cycle if sister chromatids are erroneously connected to the same pole, and are also required for correcting this error. However, the cell cycle delay, which occurs if kinetochores remain unconnected to microtubules, is largely independent of Aurora in budding yeast and human somatic cells. Contrasting with these findings, the fission yeast Aurora kinase Ark1 has been found to play a role in delaying the cell cycle when kinetochores are unattached (Petersen and Hagan, 2003). We have re-examined the phenotype of Ark1 inhibition in fission yeast mitosis and meiosis, using a temperature-sensitive allele and a small-molecule inhibitor, as well as meiosis-specific shut-down by promoter exchange. We find a clear defect in bipolar chromosome segregation in mitosis, consistent with Aurora's role in other organisms. In meiosis I, Ark1 promotes the segregation of homologous chromosomes to opposite poles. However, when recombination and side-by-side-arrangement of kinetochores are abolished, so that mitosis-like chromosomes exist in a meiosis I cell, Ark1 promotes the bi-polar segregation of sister chromatids, indicating that Ark1 is able to function independently of the underlying kinetochore morphology. Furthermore, in both mitosis and meiosis, Ark1 is required to delay the cell cycle if microtubule-kinetochore interactions occur, but not in the proper bi-polar fashion. Together, this data demonstrates that the function of Aurora kinases is highly conserved in evolution. To further reveal the mechanism of action, we have extended our analysis of Ark1 by searching for genetic interactors, which has revealed candidates of downstream effectors.

1602

**Inhibition of Aurora B Produces a Dramatic Stabilization of Kinetochore-Microtubules**

D. Cimini,[1] A. Khodjakov,[2] E. D. Salmon[1]; [1]Biology, University of North Carolina, Chapel Hill, NC, [2]Wadsworth Center, New York State Department of Health, Albany, NY

We have previously found that partical inhibition of Aurora B by 3μM ZM447439 produces a 6-fold increase in anaphase lagging chromosomes in PtK1 cells (Cimini et al., 2004, 43rd ASCB Annual Meeting). Anaphase lagging chromosomes are produced by merotelic kinetochore orientation (Cimini et al., 2001, J. Cell Biol.), in which a single kinetochore binds microtubules coming from both spindle poles rather than just one. To test the hypothesis that Aurora B inhibition results in a reduced efficiency of merotelic correction in prometaphase, we measured the changes in kMT (kinetochore-microtubule) stability in cells treated with 3μM or 20μM ZM447439. We used a PtK1 cell line expressing photoactivatable-GFP-tubulin (PA-GFP-tubulin) and measured MT poleward flux and MT turnover of photoactivated fluorescent marks across 1-3 kinetochore fibers. Because ZM447439-treated cells either spend a very short time in metaphase (3μM) or do not completely align their chromosomes (20μM), most of the cells analyzed possessed unaligned chromosomes and therefore were compared to mid- late-prometaphase untreated cells. Poleward flux of kMTs was reduced in ZM447439-treated cells as compared to untreated cells, being equal to 0.40±0.18 μm/min for 3μM and 0.42±0.16 μm/min for 20μM compared to 0.93±039 μm/min for control prometaphases. In controls, ~70% of kinetochore fiber fluorescence had a half-life of 20 sec, corresponding to non-kMT dynamics, while ~30% had a half-life of about 3 min, corresponding to kMT turnover. Both 3μM and 20μM ZM447439 did not change the fraction or turnover rate of non-kMTs, but they both substantially stablilized kMT fluorescence. These results show that inhibition of Aurora B produces a dramatic increase in kMT stability. This stability would prevent correction of merotelic attachments to the wrong pole in prometaphase, and hence induce chromosome segregation errors in anaphase detectable as lagging chromosomes. Supported by NIH GM32046

1603

**Analysis of Mad2 and Cdc20 Dynamics at Kinetochores by FLIP**

R. P. O'Quinn,[1,2] W. C. Salmon,[3] E. D. Salmon[1]; [1]Department of Biology, University of North Carolina - Chapel Hill, Chapel Hill, NC, [2]NIH-UNC Graduate Partnerships Program in Cell Motility and Cytoskeleton, Bethesda, MD, [3]Michael Hooker Microscopy Facility, University of North Carolina - Chapel Hill, Chapel Hill, NC

Faithful chromosome segregation is achieved in dividing cells with the assistance of the spindle assembly checkpoint (SAC). A complex of Mad1, Mad2, and Cdc20 forms during mitosis that prevents activation of the anaphase-promoting complex ($APC^{Cdc20}$) and mitotic progression until all chromosomes are bipolarly attached at kinetochores. Mad2 is targeted to kinetochores by Mad1, and is activated to sequester and bind Cdc20. A recently proposed "template model" of the SAC suggests that two distinct conformers of Mad2 exist in cells: a "closed" form that is bound to Mad1 or bound to Cdc20 in the checkpoint cascade, and an "open" form freely diffusing in the cytoplasm unbound to either ligand. The model predicts that 50% of Mad2 at kinetochores should be C-Mad2 stably bound to Mad1 while 50% exists as O-Mad2 that rapidly binds and releases from C-Mad2/Mad1. To test for the presence of these two distinct conformers of Mad2 at kinetochores, we used measurements of fluorescence loss in photobleaching the cytoplasm (FLIP) or fluorescence recovery after photobleaching the kinetochore (FRAP) in PtK2 cells stably expressing YFP-Mad2. For reference, we used PtK2 cells stably expressing YFP-Cdc20. Cells were treated with nocodazole to prevent microtubule interactions with kinetochores. FLIP data indicated the existence of a stable component of Mad2, averaging 28% +/- 11.3% over our 10 min measurement period. In contrast, Cdc20 exhibited no stable component. FRAP results showed on average 36% of Mad2 stable at the kinetochore over 10 min. The rapid dissociation phase for Mad2 at kinetochores had a half-life of 20s or less. We conclude that there are two distinct conformers of Mad2 at kinetochores. It is likely that the stable component is C-Mad2 and the rapidly turning over component is O-Mad2. Supported by grants from NIHGMS and HFSP.

1604

**A Polar Reeling-in Mechanism Produces the Majority of Poleward Microtubule Flux for Kinetochore Fibers in PtK1 Cells**

L. A. Cameron,[1] G. Yang,[2] D. Cimini,[1] J. C. Canman,[1] O. Kisurina-Evgenieva,[3] A. Khodjakov,[3,4] E. D. Salmon[1]; [1]Dept of Biology, Univ. of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Laboratory for Compuatational Cell Biology, Dept. of Cell Biology, The Scripps Research Institute, La Jolla, CA, [3]New York State Department of Health, Wadsworth Center, Albany, NY, [4]Department of Biomedical Sciences, State University of New York, Albany, NY

Poleward microtubule flux is produced by forces generated within the mitotic spindle that help segregate chromosomes and produce tension on kinetochores. Translocation of tubulin polymer poleward coupled to minus end depolymerization near the spindle poles constitutes poleward flux. Three mechanisms may drive poleward flux. In a bipolar spindle, the sliding of microtubules (MTs) toward their poles could be produced by (1) motor proteins like kinesin 5 that cross-link and slide overlapping, oppositely-oriented MTs or by (2) kinesins bound to chromosome arms. (3) Minus-end depolymerization at the poles could be coupled to reeling-in of MTs in bipolar and monopolar spindles. We have used quantitative fluorescent speckle microscopy and fluorescence photomarking methods to determine the contribution of sliding and reeling-in to poleward flux of kinetochore MTs (kMTs) in mitotic PtK1 cells. When kinesin 5 is inhibited, poleward flux of kMTs in either monopolar (no anti-parallel MTs) or bipolar spindles occurs at ~75% of the normal rate in a bipolar spindle. This indicates that, polar reeling-in is the dominant mechanism of kMT flux in mammalian tissue cell mitosis.

1605

**Analysis of Kinetochore-lesions That Promote Chromosome Missegregation but Are Not Monitored by Spindle Checkpoint Proteins**

V. M. Draviam,[1] F. Stegmeier,[2] I. Shapiro,[1] B. Aldridge,[1] S. Elledge,[3] P. Sorger[1]; [1]Biology, MIT, Cambridge, MA, [2]Biology, Harvard Medical School, Boston, MA, [3]Harvard Medical School, Boston, MA

Unattached kinetochores recruit the spindle checkpoint proteins (Mad1-2, Bub1, BubR1&Mps1). Spindle checkpoint proteins pause anaphase onset until the kinetochore-microtubule attachment is complete and thus prevent missegregation. We report two lines of evidence that show chromosome missegregation despite the normal recruitment of spindle checkpoint proteins to kinetochores. We report novel lesions in kinetochore orientation generated by depleting the MAPs EB1 or APC. These lesions cause missegregation but these are not sensed by spindle checkpoint. By tracking the history of individual centromeres in live-cells, we show that unperturbed cells orient their centromeres in an orderly fashion. In contrast, EB1 or APC depleted cells display centromeres that are under insufficient tension and disordered orientation; misoriented centromere undergo missegregation. Overexpression of EB1 mutants that fail to bind to APC promote similar errors in segregation. Missegregations due to lesions in orientation are particularly interesting, since they fail to invoke a checkpoint arrest in checkpoint-proficient cells. This is in contrast lesions generated in the absence of CLIP170, chTOG1, LIS1 or Dynein, which recruit checkpoint proteins and arrest cells. In summary, perturbing the function of EB1 or APC promotes missegregation, without abrogating the recruitment of checkpoint proteins to kinetochores. A similar missegregation phenotype is observed in the absence of a kinase required to arrest cells in the presence of Taxol (RTF1). RTF1 identified in the Elledge lab from an RNAi-based screen for Taxol-induced arrest. In the absence of RTF1, cells undergo missegregation despite normal recruitment of Mad and Bub proteins - a status similar to EB1 or APC depletions. In summary, because missegregation in these two cases occur despite normal recruitment of spindle checkpoint proteins, we propose that spindle checkpoint independent mechanisms might play a role in accurate segregation of chromosomes.

1606

**Aurora B Kinase and MCAK Are Preferentially Localized to Points of Merotelic Microtubule Attachments**

A. K. McCullough, W. Lan, T. Stukenberg; Biochemistry, UVA, Charlottesville, VA

Proper chromosome segregation during mitosis is essential for viability, and is ensured by bipolar microtubule attachment on sister kinetochores. Often during mitosis, kinetochores form merotelic attachments in which a single kinetochore is attached to microtubules from both spindle poles. The microtubule depolymerizing Kin-I kinesin MCAK regulates Aurora B kinase at kinetochores, and may play a role in releasing improper microtubule attachments during prometaphase. The outer kinetochore plate protein Ndc80 (and its associated complex) is necessary for proper chromosome segregation and checkpoint activation, and is hypothesized to be the site of microtubule end-on attachment to kinetochores. We have used three channel confocal imaging to examine merotelic attachments in *Xenopus* S3 cells. In merotelically attached kinetochores, we find the expected two sister kinetochore populations of Ndc80 and novel foci in the inner centromere region. Microtubules from both poles emanate from these Ndc80 foci suggesting that this is the point of merotelic attachment. We have systematically identified proteins whose levels are increased at these points of merotelic attachment. MCAK and Aurora B are preferentially localized to these stretched kinetochores, and immunofluorescence with phosphoantibodies suggests that Aurora B is regulating this population of MCAK. These data support the hypothesis that Aurora B-regulated MCAK is responsible for the release of microtubules from merotelically attached kinetochores. We also conclude that the outer kinetochore plate is a malleable region that can be distorted by microtubule forces.

1607

**HURP, a Spindle-Associated Protein, Promotes Chromosome Congression and Tension Across Sister Kinetochores by Modulating Microtubule Dynamics**

J. Y. Wong, G. Fang; Department of Biological Sciences, Stanford University, Stanford, CA

Accurate chromosome segregation during mitosis requires the assembly of the mitotic spindle that captures all kinetochores by spindle microtubules, aligns chromosomes along the metaphase plate, and establishes tension across the sister kinetochores. To identify key regulators in these processes, we performed a targeted functional genomic screen for proteins required for efficient chromosome congression. From this screen, we identified the hepatoma up-regulated protein (HURP), originally reported as a gene product over-expressed in liver cancer cells. We found that HURP is a spindle-associated protein that preferentially localized to chromosome-proximal regions of the mitotic spindle. siRNA-mediated depletion of HURP results in a fifteen-fold increase in the number of metaphase cells with misaligned chromosomes that arise from the lack of kinetochore capture by spindle microtubules. HURP-depleted cells at metaphase also exhibit less tension across sister kinetochores on chromosomes that have congressed to the metaphase plate. Biochemically, HURP binds directly to microtubules, facilitates microtubule polymerization, stabilizes the mitotic spindle, and promotes timely bipolar spindle formation. We conclude that HURP, by modulating spindle microtubule dynamics, is required for efficient capture of kinetochores by spindle microtubules and for the establishment of proper tension across sister kinetochores. Furthermore, over-expression of either full-length HURP or its microtubule-binding domain hyper-stabilizes spindle microtubules, alters spindle morphology, and reduces tension across sister kinetochores at metaphase. Thus, the precise physiological level of HURP protein is critical for maintaining a functional mitotic spindle that generates proper pulling forces across sister kinetochores. We speculate that over-expression of HURP in tumor cells changes the dynamics of the mitotic spindle and reduces the fidelity of chromosome segregation

during mitosis.

1608

**Regulation of Kinetochore Fiber Elongation by Dynein**

U. S. Tulu, N. P. Ferenz, P. Wadsworth; Biology, University of Massachusetts, Amherst, MA

To examine mitotic spindle formation in mammalian cells, we developed a method to monitor microtubule assembly separately at centrosomes and chromosomes. LLCPK1 cells expressing GFP-tubulin are treated with nocodazole, partially released with drug-free media, and observed with spinning disc confocal microscopy. Under these conditions, microtubules form first at centrosomes and subsequently at discrete locations on chromosomes that stain for the kinetochore protein Bub1. Kinetochore-associated microtubules rapidly coalesce into microtubule foci. Injection of anti-dynein antibodies blocks coalescence and results in a dramatic elongation of kinetochore-associated microtubules. Centrosomal microtubules are not detectably altered following these injections. We tested the possibility that elongation of kinetochore-associated microtubules results from the mislocalization of KinI kinesins following inhibition of dynein, as recently documented in spindles assembled in frog extracts (Gaetz and Kapoor, 2004, JCB). Preliminary immunolocalization experiments demonstrate that Kif2a remains localized to kinetochore-associated microtubules in cells injected with anti-dynein antibodies. Microinjection of polyclonal antibodies directed at Kif2a, or MCAK, (kind gifts of Drs. D. Compton and C. Walczak, respectively) induced elongation of centrosomal microtubules, and spindle formation, but did not phenocopy the anti-dynein antibody effect. These results demonstrate that centrosomal microtubule elongation is regulated by KinI kinesins, whereas kinetochore-associated microtubule elongation is regulated by dynein. Photoactivation experiments are being used to determine the location of kinetochore-fiber elongation in this system. These data show that dynein/dynactin complexes at the kinetochore regulate elongation of kinetochore-associated microtubules in mammalian cells.

1609

**Kinetochore Fibrils: Slender Connections between Chromatin and Spindle Microtubules Revealed by EM Tomography of Rapidly Frozen, Freeze-Substitution Fixed Cells**

R. McIntosh, M. K. Morphew, E. T. O'Toole, D. N. Mastronarde; Dept. of M.C.D. Biology, Univ. Colorado, Boulder, CO

Electron microscope tomography has been used to examine the structure of the links between spindle microtubules (MTs) and the chromosomes of three species. Mammalian cells, strain $PtK_1$, and both fission and budding yeasts were cryo-immobilized by rapid freezing at high pressure, fixed by freeze-substitution, embedded in plastic, sectioned at ~300 nm, stained, then imaged in an electron microscope at either 300 or 750 KeV. Images of mitotic cells were collected at 1 - 1.5$^o$ tilt intervals around two orthogonal axes over $\pm$ 60$^o$. Three-dimensional (3D) reconstructions were generated by back-projection, and the resulting images were viewed and modeled with the IMOD software package (see http://bio3d.colorado.edu). Mitotic chromosomes and their sites of attachment to spindle MTs were identified in 3D, and the Slicer feature of IMOD was used to extract the volume surrounding each kinetochore microtubule end. The images showed fibrils 2 - 4 nm in diameter that extended from the centromeric heterochromatin out into the region of the MT ends. The fibrils made apparent contacts with the ends of the MT protofilaments or with either the inside or the outside of MT walls. The fibrils are slender and sinuous, so they are difficult to see, except in very thin slices of well-fixed spindles, such as the 2 - 10 nm slices that can be extracted from tomograms. We interpret these fibrils as fibrous protein complexes that comprise some of the kinetochore proteins in these organisms. We speculate that the connections to different places on the MTs correspond to different molecular links between the chromosome and the spindle.

1610

**Molecular Requirements for Microtubule Formation at Kinetochores and Centrosomes Using siRNA**

U. S. Tulu, C. J. Fagerstrom, P. Wadsworth; Biology, University of Massachusetts, Amherst, MA

Microtubules are a major component of a bipolar spindle. To examine microtubule assembly, we developed an assay in which microtubule formation around chromosomes and centrosomes can be visualized separately. LLCPK1 cells expressing GFP-tubulin are treated with nocodazole to depolymerize all microtubules, washed to remove drug and spinning disk confocal microscopy was used to visualize microtubule assembly at centrosomes and at chromosomes. We used siRNA to identify molecular requirements of microtubule formation. These experiments, which were performed in HeLa cells, have shown that TPX2, one of the targets of Ran pathway, and survivin, a member of chromosomal passenger complex, are involved in assembly and stability of microtubules around chromosomes. To further investigate the functions of these proteins in living cells with flat morphology, we are using siRNA in LLCPK1 cells, following partial release from nocodazole. We first synthesized siRNA's (Dharmacon) against LLCPK-Survivin (pig-Survivin), whose complete cDNA is available from NCBI. pig-Survivin was co-transfected with fluorescently labeled siRNA s to identify the transfected cells. Following partial nocodazole release, microtubule formation around chromosomes was reduced, while no change in centrosomal microtubules was observed, confirming the phenotype observed in HeLa cells. In order to deplete gene products whose complete sequence is not available, we designed PCR primers using known EST sequences. Our first candidate, pig-TPX2 was sequenced completely following this procedure. Sequence analysis of pig-TPX2 showed that it shares 89% sequence identity to hsTPX2 at DNA level and 87% sequence identity at protein level. These experiments demonstrated that we can use siRNA to downregulate protein levels in LLCPK1 cells and that this approach will allow us to identify molecular requirements of microtubule formation at kinetochores and centrosomes separately in real time.

1611

**TOGp Depletion Increases Tension at Sister Kinetochores and Suppresses Kinetochore Directional Instability**

B. E. Becker, J. Morabito, L. Cassimeris; Department of Biological Sciences, Lehigh University, Bethlehem, PA

TOGp is the human member of the XMAP215 family of MAPs that is required for proper mitotic spindle assembly. Previous results showed that TOGp plays an important role in spindle pole organization, however, its affects on other aspects of spindle assembly and function have not been addressed. We used siRNA knockdown of TOGp in cultured cells to investigate TOGp function in spindle microtubule dynamics and kinetochore motility. FRAP of GFP-tubulin expressing cells demonstrated that the turnover of non-kinetochore microtubules is not altered in TOGp-depleted cells. The rate of microtubule plus end elongation, measured in cells expressing GFP-EB1, were equal in siRNA and sense RNA treated cells.

Together these results indicate that TOGp is not required for non-kinetochore microtubule dynamics. However, examination of sister chromatid separation in TOGp-depleted cells stained with CREST or the kinetochore marker CENP-E showed that the distance between sister kinetochores increased, indicating that kinetochores are under greater tension compared to control cells. The loss of tension at sister kinetochores suggests that there are defects in microtubule flux or in the coordination of sister kinetochore movements, or both. Preliminary observations of kinetochore motility in TOG-depleted cells expressing GFP-CENP-A demonstrated a significant decrease in kinetochore motility after TOGp depletion. Our results suggest that TOGp is required to coordinate sister kinetochore motility and/or generate kinetochore movement away from the pole (AP); either defect may explain why TOGp-depleted chromosomes often fail to bi-orient. Experiments are underway to examine kinetochore microtubule flux in TOG-depleted cells to determine whether the defects in kinetochore motility result from changes in flux.

1612

**Reduced Microtubule Dynamics Contribute to Loss of Tension at the Kinetochore**

M. K. Gardner,[1] C. G. Pearson,[2] L. V. Paliulis,[3] D. J. Odde,[1] K. Bloom[3]; [1]Biomedical Engineering, University of Minnesota, Minneapolis, MN, [2]MCDB, University of Colorado, Boulder, CO, [3]Biology, University of North Carolina, Chapel Hill, NC

Sister kinetochores in budding yeast metaphase spindles are bioriented and separated into a metaphase configuration via kinetochore-attached microtubules (kMTs). Although kMTs are dynamic during yeast metaphase, individual plus-end behavior cannot be resolved, and the role of dynamic kMTs in maintaining tension generated via the stretch of chromatin between sister kinetochores is unclear. In addition, although tension has been implicated in the establishment of bipolar attachment at the kinetochore, the effect of moderate tension loss on the fidelity of chromosome segregation has not been established. Here, we have examined kMT plus-end behavior and kinetochore clustering in a β-tubulin mutant with reduced microtubule dynamics. Through the use of 1) high temporal and spatial resolution GFP-Tub1 fluorescence recovery after photobleaching (FRAP) experiments, 2) quantitative analysis of kinetochore-associated Cse4-GFP fluorescence, and 3) computational simulations of these experiments, we find that slowed kMT plus-end dynamics result in a ~20% reduction in separation between sister kinetochore clusters, which is likely due to restricted towards-the-pole excursions of the kMT plus-ends. We find that this moderate loss in tension at the kinetochore results in significant chromosome loss, indicating that robust kMT dynamics are instrumental in establishing tension, and that a minimal level of tension is required to ensure accurate chromosome segregation.

1613

**Comparative Proteomic and Functional Analysis of *C elegans* and Human Kinetochores**

I. M. Cheeseman,[1] S. L. Kline-Smith,[1] I. McLeod,[2] J. R. Yates,[2] T. Fukagawa,[3] A. Desai[1]; [1]Ludwig Institute for Cancer Research, La Jolla, CA, [2]The Scripps Research Institute, La Jolla, CA, [3]National Institute of Genetics and SOKENDAI, Mishima, Japan

The kinetochore is a large proteinaceous structure that serves as the primary chromosome attachment site for spindle microtubules. Identification of the full complement of metazoan kinetochore components remains an important challenge. Although more than 60 budding yeast kinetochore components have been identified, the absence of catalytic domains in the majority of these proteins and high sequence divergence has limited bioinformatics-based identification of metazoan homologues. We have conducted systematic proteomic analysis to define the composition of diffuse *C. elegans* kinetochores and localized human kinetochores. This work has identified 8 novel *C. elegans* kinetochore proteins and 10 novel human kinetochore proteins. Comparison of the purifications revealed both similar kinetochore sub-complexes in *C. elegans* and humans, as well as sub-complexes that are specific to the human kinetochore. The former class includes the KNL-1/3 protein network with NDC80/Hec and MIS-12 sub-complexes. The latter class includes CENP-H/CENP-I and 6 novel associated proteins. By analyzing the consequences of depleting newly identified proteins in both systems, we have defined the contributions that they make to kinetochore assembly and function. This approach has revealed striking similarities between *C. elegans* and human cells in the localization dependencies of the sub-complexes present in both systems. In contrast, the proteins not present in *C. elegans* represents a distinct branch of human kinetochore assembly. Phenotypic analysis in human cells has revealed an important role for both sets of proteins in assembly of kinetochore fibers and chromosome alignment and segregation. In total, this comparative dissection of kinetochores in two highly divergent metazoans is helping decipher the composition, assembly, and function of this key cell division organelle.

1614

**Dynamic Localization of Aurora-C and Aurora-B during Male and Female Mouse Meiosis**

C. C. Tang, C. Lin, T. K. Tang; Inst. Biomedical Sciences, Academia Sinica, Taipei, Taiwan Republic of China

The Aurora kinases play essential roles in many aspects of cell division including the control of centrosome function, kinetochore-microtubule interactions, and cytokinesis. In mammals, three distinct Aurora kinase members (Aurora-A, -B, and -C) have been identified. We have generated both a polyclonal and a monoclonal antibody that specifically against Aurora-C. The localization of Aurora-C was analyzed by immunofluorescence staining on chromosome spreads of mouse spermatocytes or in squashed seminiferous tubules. Aurora-C was first detected as clusters of chromocenters in diplotene spermatocytes and was concentrated at centromeres in metaphase I and II. Interestingly, Aurora-C was also found along the chromosome axes, including both the regions of centromeres and the chromosome arms in diakinesis. During the anaphase I/telophase I and anaphase II/telophase II transitions, Aurora-C was relocalized to the spindle midzone and midbody. A similar distribution pattern was also observed for Aurora-B during male meiotic divisions. Interestingly, we detected no Aurora-C in mitotic spermatogonia and in the examined normal mouse somatic tissues or cell lines suggesting that Aurora-C is a meiotic chromosomal passenger protein. Furthermore, immunoprecipitation analysis revealed that INCENP associated with Aurora-C in the male testis, suggesting that INCENP may recruit Aurora-C (or some other factor(s) may recruit INCENP and Aurora-C) to meiotic chromosomes. We also analyzed the subcellular localization of Aurora-B and -C during oocyte meiotic maturation. The localization pattern of Aurora-C in female oocytes is similar to that observed in male spermatocytes. Surprisingly, we detected no Aurora-B in female oocytes either by immunoblotting or immunofluorescence analysis. Together, our findings suggest that Aurora-C appears to be mainly expressed in meiotic germ cells and may play essential roles during meiosis.

1615

**Inhibition of Microtubule Flux and Other Effects of Monastrol on Crane-fly Spermatocytes**

J. R. LaFountain,[1] C. S. Cohan[2]; [1]Biological Sciences, University at Buffalo, Buffalo, NY, [2]Pathology and Anatomy, University at Buffalo, Buffalo, NY

One model for the poleward flux of microtubules in mitotic spindles requires plus end-directed motors that slide anti-parallel microtubules of the two half spindles away from the equator and toward the two spindle poles. To test this model in crane-fly spermatocytes, which exhibit robust flux in their kinetochore fibers, we treated isolated testes with monastrol (Sigma-Aldrich), an inhibitor of vertebrate bipolar kinesin Eg5, and then ruptured testes under oil to assess flux by fluorescent speckle microscopy. 100µM monastrol, a saturating dose in vertebrate systems, had no detectable effect on spermatocytes, even after incubations of 3 hrs. 1mM monastrol arrested meiosis, and upon microinjection of rhodamine-tubulin into spermatocytes arrested in metaphase, there was no detectable rh-tubulin incorporation into microtubules, to be expected if flux is turned off by monastrol. The arresting effect of monastrol on spermatocytes is reversible in fresh insect buffer. An interpretation in terms of monastrol-specific inhibition of Eg5 is complicated, however, by other effects of monastrol none of which are readily explained simply by its action on an Eg5 homologue. For example, monastrol caused arrest of cells undergoing cytokinesis. In addition, cells in late diakinesis failed to undergo nuclear envelope breakdown, although DMSO-treated controls invariably underwent NEB within 1 hr of initial observation. Thus, a motor-based mechanism of flux may indeed be operative in spermatocytes. However, in view of the observed other apparently non-Eg5-specific effects of monastrol on these meiotic cells, additional mechanisms, e.g., intrinsic treadmilling of microtubules, cannot be ruled out, because such alternatives also could be monastrol sensitive, albeit indirectly. This study has demonstrated that monastrol is not as widely applicable for testing the role of motors in microtubule flux as it appears to be in vertebrate systems.

1616

**Effects of Jasplakinolide on Chromosome Movement in Crane Fly Spermatocytes**

L. Xie, A. Forer; Biology, York University, Toronto, ON, Canada

Previous experiments utilized the actin inhibitors cytochalasin D and latrunculin to implicate actin in anaphase chromosome movements (e.g. Forer and Pickett-Heaps 1998): these inhibitors cause depolymerization of actin and cause anaphase chromosomes to slow or to stop moving. In our experiments we use a stabilizer of actin filaments, jasplakinolide, at 0.55mM or 1.1mM, to probe the role of actin in anaphase chromosome movements in crane-fly spermatocytes. We treated living crane-fly spermatocytes at various stages of meiosis I and studied the effects on chromosome movements. We describe the effects on individual chromosomes rather than on cells because not all chromosomes in the cell responded the same. When we added jasplakinolide in anaphase, chromosomes generally slowed or stopped moving (34/46), though stopping was only temporary. Some chromosomes moved normally in the presence of jasplakinolide (11/46) and one accelerated. Jasplakinolide added in prometaphase or metaphase caused a majority of the chromosomes (19/32) to stop or to move slowly during subsequent anaphase, and some chromosomes that stopped did not resume movement; the remaining chromosomes (13/32) moved normally. In initial experiments (in three cells) we added jasplakinolide at or before the time of *nuclear membrane breakdown*: in one cell the jasplakinolide blocked chromosome attachment to the spindle; in the other two cells about half the chromosomes moved normally and half stopped or slowed during anaphase. All these experiments indicate that spindle function is disrupted when jasplakinolide stabilizes actin filaments. To try to understand why, we will use confocal microscopy to study the disposition of actin filaments in these cells.

1617

**A Role for the Anaphase Promoting Complex Inhibitor Emi2/XErp1, a Homolog of Emi1, in Cytostatic Factor Arrest of Xenopus Eggs**

J. Tung, D. Hansen, K. Ban, A. Loktev, M. Summers, P. Jackson; Stanford, Stanford, CA

Unfertilized vertebrate eggs are arrested in metaphase of meiosis II (MII) with high cyclin B/Cdc2 activity to prevent parthenogenesis. Until fertilization, exit from metaphase is blocked by an activity called cytostatic factor (CSF), which stabilizes cyclin B by inhibiting the Anaphase Promoting Complex (APC) ubiquitin ligase. The APC inhibitor Emi1 was recently found to be required for maintenance of CSF arrest. We show here that exogenous Emi1 is unstable in CSF-arrested *Xenopus* eggs and is destroyed by the $SCF^{\beta TrCP}$ ubiquitin ligase, suggesting that endogenous Emi1, an apparent 44 kDa protein, requires a stabilizing factor. However, anti-Emi1 antibodies cross-react with native Emi2/Erp1/FBXO43, a homolog of Emi1 and conserved APC inhibitor. Emi2 is stable in CSF-arrested eggs, is sufficient to prevent CSF release, and is rapidly degraded in a Polo like kinase 1-dependent manner in response to calcium-mediated egg activation. These results identify Emi2 as a CSF maintenance protein.

1618

**The Cytostatic Factor Emi2 is the Critical Target of Calcium/Calmodulin-dependent Kinase II and Polo-like Kinase 1 in Exit from Metaphase-II of Meiosis**

D. V. Hansen,[1] J. J. Tung,[1] P. K. Jackson[2]; [1]Program in Cancer Biology, Stanford Univ. School of Medicine, Stanford, CA, [2]Pathology and Microbiology & Immunlogy, Stanford Univ. School of Medicine, Stanford, CA

In vertebrate meiosis, unfertilized eggs are arrested in metaphase-II by cytostatic factor (CSF), which is required to maintain mitotic cyclin-dependent kinase activity. Fertilization triggers a transient increase in cytoplasmic free $Ca^{2+}$, causing CSF inactivation and ubiquitin-dependent destruction of mitotic cyclin by the Anaphase-Promoting Complex (APC). Previous studies have shown that calcium/calmodulin-dependent kinase II (CaMKII) and the Polo-like kinase Plx1 are essential factors for $Ca^{2+}$-induced meiotic exit, but the critical targets of these kinases are unknown. We recently characterized the APC inhibitor Emi2/Erp1 as a pivotal component of CSF. Upon fertilization, a degron in Emi2 is phosphorylated by Plx1 and then recognized by the $SCF^{\beta TrCP}$ ubiquitin ligase, allowing ubiquitination-dependent destruction of Emi2, APC activation, and meiotic exit. An important question is how the increase in $Ca^{2+}$ targets Plx1 towards Emi2, especially since Plx1 is active before fertilization. We find that inhibition of CaMKII prevents $Ca^{2+}$-induced Emi2 destruction, and that a dominant, activated CaMKII drives Emi2 destruction in the absence of $Ca^{2+}$. Moreover, phosphorylation of Emi2 by CaMKII induces a strong interaction between Emi2 and the PoloBox domain of Plx1, allowing subsequent phosphorylation of the Emi2 degron by Plx1 and recruitment of βTrCP. A specific motif in Emi2 contains consensus sites for phosphorylation by CaMKII and, when phosphorylated, Plx1 recruitment. Mutation of this motif in Emi2 causes failure to recruit Plx1 and failure to be destroyed in response to $Ca^{2+}$. Thus, the CSF component Emi2 is a critical and direct target for both CaMKII and Plx1, providing the first

detailed molecular mechanism explaining how CaMKII and Plx1 coordinately direct the activation of the APC and meiotic exit upon fertilization. Evidence for an independent mode of Emi2 inactivation will be presented.

## Kinetochores (1619-1644)

1619

### Kinetochore Ultrastructure of *Drosophila* S2 Cells Previously Observed in Life

H. Maiato,[1,2] P. Hergert,[2] Y. Dong,[2] C. Rieder,[2] B. McEwen[2]; [1]Institute for Molecular and Cell Biology, Porto, Portugal, [2]Laboratory of Cell Regulation, Division of Molecular Medicine, Wadsworth Center, New York State Department of Health, Albany, NY

Cell cultures of *Drosophila melanogaster* are widely used as a model system for the molecular dissection of mitosis in animals. However, little is known about the ultrastructure of the mitotic apparatus in these cells, in particular, the kinetochore and its microtubules (MTs). To obtain a detailed ultrastructural analysis of the kinetochore and kinetochore-fiber in S2 cells we developed a correlative light and electron microscopy (EM) approach. We followed S2R+ cells, an S2 clone which adheres to glass surfaces in the absence of any coating, by DIC time-lapse light microscopy as they entered mitosis. When most chromosomes were aligned at the spindle equator the cells were fixed, circled with a diamond scribe and processed for serial section EM. Direct MT counts from 31 kinetochores derived from 3 serially sectioned metaphase cells gave an average distribution of 11 ± 2 MTs per kinetochore. These numbers contrast with the numbers of 4-6 MTs per kinetochore that are reported for *Drosophila* spermatocytes. In addition, we sought to characterize the ultrastructure of the *Drosophila* mitotic kinetochore deprived of microtubules by treating S2R+ cells with colchicine. When compared with the vertebrate kinetochore, we found striking differences in kinetochore morphology. Overall, the results of this study provide important information needed for interpreting subsequent live cell studies on *Drosophila* cell cultures, particularly those involving the introduction of fluorescence combined with RNAi.

1620

### Role of Spindle Checkpoint Protein ZW10 in Chromosome Segregation

I. M. Shapiro, V. M. Draviam, P. K. Sorger; Biology, MIT, Cambridge, MA

The spindle assembly checkpoint ensures accurate segregation of chromosomes by monitoring the formation of bipolar attachments by paired sister chromatids to kinetochore fibers. When unattached or maloriented chromatids are present, the checkpoint delays anaphase onset, increasing the time available for completion of kinetochore-microtubule binding. The checkpoint involves a group of well conserved proteins, including Mad1-3, Bub1, Bub3, and Mps1 as well as several proteins such as ZW10 (Zeste-white 10), ROD (Rough deal) and Zwilch, that are specific to metazoans and whose precise functions remain unknown. Given the high degree of conservation of Mad and Bub proteins from yeast to man, it is of considerable interest to determine the role of checkpoint proteins that are specific to higher eukaryotes. To investigate the role of ZW10 in chromosome segregation and checkpoint control we have performed RNAi-mediated depletion of ZW10 in HeLa cells and then followed them by live-cell imaging. We find that depletion of ZW10 causes checkpoint failure and errors in chromosome segregation. These errors manifest themselves during metaphase as a failure of congression and during anaphase as lagging chromatids. We find that many kinetochore fibers in ZW10-depeleted cells are unstable, and unlike normal kinetochore fibers, depolymerize in the cold. Several important kinetochore microtubule-binding proteins are absent from ZW10-depleted kinetochores, possibly explaining this instability. Ongoing studies are aimed at determining why these lesions do not arrest cell division.

1621

### CENP-A Deposition Occurs De Novo and Independently of DNA Replication Following Fertilization in C elegans

F. E. Hyndman,[1] P. S. Maddox,[1] J. Monen,[1] S. Ward,[2] K. Oegema,[1] A. Desai[1]; [1]LICR, UCSD, La Jolla, CA, [2]Molecular and Cellular Biology, University of Arizona, Tuscon, AZ

Nucleosomes in which the histone variant CENP-A replaces H3 serve as the structural foundation for kinetochore assembly and are proposed to be an epigenetic mark that propagates centromere identity. Quantitative retention of CENP-A nucleosomes during mammalian spermatogenesis, despite removal of H3 nucleosomes, suggested that this chromatin domain is transmitted through fertilization. However, a role for sperm-derived CENP-A in propagating centromere identity has not been demonstrated. We have analyzed CENP-A deposition following fertilization in *C. elegans*, an organism with holocentric chromosomes that form diffuse kinetochores. ~30 min after fertilization, sperm and oocyte chromosomes have replicated and formed CENP-A chromatin domains on opposing poleward faces. Quantitative immunoblotting revealed that *C. elegans* sperm have essentially no CENP-A (<300 copies per sperm), in contrast to ~300,000 copies per embryonic nucleus. *In vivo*, no CENP-A is detected on wild-type sperm chromosomes introduced into CENP-A depleted oocytes. Thus, CENP-A on sperm chromosomes is recruited *de novo* after fertilization. We also analyzed oocyte nuclei, which undergo meiosis after sperm entry. Surprisingly, CENP-A is removed from chromosomes after meiosis I and does not reappear until after meiosis II is complete, indicating that CENP-A deposition on oocyte chromosomes also occurs *de novo.* CENP-A deposition is replication-independent in mammalian cells. To test if the same is true in *C. elegans*, we depleted CDT1 and CDC6, two proteins essential to initiate replication. DAPI fluorescence was decreased by half and chromosomes did not have paired sister chromatids, but CENP-A incorporation, and subsequent targeting of outer kinetochore proteins, was still detected. These results indicate that CENP-A deposition following fertilization in *C. elegans* occurs *de novo* albeit independently of DNA replication. Thus CENP-A nucleosomes do not propagate an epigenetic mark through fertilization in this system.

1622

### Probing Kinetochore Assembly in *Xenopus* egg Extracts

K. J. Milks, A. F. Straight; Biochemistry, Stanford University, Stanford, CA

Kinetochores are responsible for attaching chromosomes to the mitotic spindle, monitoring proper chromosome alignment, and coupling chromosomes to spindle forces during mitosis. Kinetochore proteins can be divided into two classes: those that constitutively mark the centromere throughout the cell cycle, and those that assemble only during mitosis to form an active mitotic kinetochore. Two constitutive kinetochore proteins that are required for both interphase and mitotic kinetochore function are the histone H3 variant centromere protein A (CENP-A) and the

centromere binding protein centromere protein C (CENP-C). We are using the *Xenopus laevis* egg extract system to ask how CENP-C is recruited to centromeric chromatin during interphase and how it contributes to kinetochore assembly in mitosis. We have isolated the *X. laevis* homolog of CENP-C, which colocalizes with CENP-A at centromeres of cultured cells and in nuclei assembled in egg extracts. Immunolocalization of CENP-A in artificially-decondensed *Xenopus* sperm indicates that *Xenopus* CENP-A is maintained in chromatin throughout meiosis but that CENP-C is removed. Upon incubation of sperm in mitotic or interphase egg extract, CENP-C is loaded at the centromere. Furthermore, highly clarified egg extracts support CENP-C assembly onto sperm chromatin. We are using this *in vitro* assembly assay to explore the mechanisms of CENP-C assembly onto centromeres and to determine its role in the higher order assembly of mitotic kinetochores.

1623

**A Myb Domain Protein Directs Assembly of CENP-A Containing Centromeric Chromatin in the *C elegans* Embryo**

P. S. Maddox, F. Hyndman, K. Oegema, A. Desai; LICR, UCSD, La Jolla, CA

Nucleosomes containing the histone H3 variant CENP-A form the structural foundation for kinetochore assembly and are proposed to act as an epigenetic mark for centromere identity. We are investigating the mechanism of CENP-A deposition in the one-cell stage *C. elegans* embryo. Depletion of CENP-A in this system results in a "kinetochore null" phenotype characterized by a severe chromosome segregation defect, inability to assemble a mechanically stable spindle, and failure to target all other kinetochore proteins to chromosomes. By re-screening embryonic lethal genes identified in genome-wide RNA interference (RNAi) screens, we have identified KNL-2, a divergent Myb domain-containing protein, as uniquely required for CeCENP-A chromatin assembly. Depletion of KNL-2 prevents CeCENP-A localization to chromosomes without affecting the amount of CeCENP-A protein. KNL-2 depleted embryos exhibit a kinetochore null phenotype and a chromosome condensation defect similar to CeCENP-A depleted embryos. KNL-2 co-localizes with CeCENP-A throughout the cell cycle and depletion of CeCENP-A disrupts KNL-2 targeting. Depletion of KNL-2 in post-embryonic divisions resulted in severe developmental defects and lethality, indicating that KNL-2 function is not restricted to the one-cell stage embryo. KNL-2 and CeCENP-A co-fractionate in nuclei prepared from embryos. Together, our results show that KNL-2 is specifically required for assembly of CeCENP-A chromatin and consequently for kinetochore assembly and chromosome segregation. We hypothesize that KNL-2 binds DNA directly via its divergent Myb domain, and acts in concert with CeCENP-A to mark centromeric regions on chromosomes and form the specialized centromeric chromatin structure required for kinetochore assembly and function.

1624

**Investigating the Role of a CHD-Family Chromatin Remodeling Complex in the Establishment of Centromeric Chromatin Structure**

I. M. Brennan, A. F. Straight; Department of Biochemistry, Stanford University, Stanford, CA

Centromeric chromatin in all eukaryotes is marked by the incorporation of nucleosomes containing CENP-A in lieu of histone H3. Evidence from multiple organisms indicates that CENP-A-containing chromatin has both a unique higher-order structure and a pattern of covalent histone amino-terminal tail modifications distinct from surrounding heterochromatin. Though the mechanism of CENP-A loading and centromeric chromatin establishment is unknown, it is likely that chromatin remodeling complexes play a role in specification of centromeric chromatin structure. By affinity chromatography in Xenopus egg extract we have identified a CHD-family histone deacetylase and ATP-dependent chromatin remodeling complex that preferentially binds to the amino-terminal tail of CENP-A. We have investigated the role of this complex in CENP-A loading and maintenance in Drosophila and human cell lines as well as a Xenopus egg extract system. Depletion of each member of the complex by RNA interference or immunodepletion from Xenopus extract do not influence the level of CENP-A at the centromere. This result suggests that the CHD complex is not directly involved in CENP-A loading. We are currently investigating whether the CHD complex is recruited to the centromere by specific binding to CENP-A and plays a role in establishing the higher-order structure of centromeric chromatin and pericentric heterochromatin.

1625

**Characterization of a Novel Kinetochore Protein Scm3p in *Saccharomyces cerevisiae***

M. Hou,[1] K. T. Simons,[2] C. W. Espelin,[1] S. C. Harrison,[2] P. K. Sorger[1]; [1]Biology, Massachusetts Institute of Technology, Cambridge, MA, [2]Biological Chemistry and Molecular Pharmacology and Pediatrics, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA

Chromosome segregation at mitosis is crucial for maintenance of genetic integrity in eukaryotic cells. To ensure high-fidelity chromosome segregation, the accurate assembly of kinetochores and their stable attachment to microtubules are necessary. Recent studies in *Saccharomyces cerevisiae* have shown that kinetochores are complex proteinaceous structures containing at least 50 protein components. The assembly of this large structure begins with the binding of CBF3 directly to the essential CDEIII element of CEN DNA. CBF3 contains four protein subunits, Ndc10p, Cep3p, Ctf13p and Skp1p, but it has been difficult to determine the precise role of CBF3 in kinetochore formation. Despite the recent explosion in the number of known kinetochore subunits, few Ndc10p, Cep3p or Ctf13p binding proteins are known. Here we describe the characterization of a new Ndc10p binding protein Scm3p (suppressor of chromosome missegregation). Scm3p is essential for cell viability that has previously been identified as a high copy suppressor of *cse4-1*. We find that Scm3p interacts with Ndc10p *in vivo* and *in vitro* and that, in living cells, it exhibits a bilobed localization typical of core kinetochore components. By ChIP, Scm3p is specifically bound to CEN DNA and we have been able to reconstitute this interaction *in vitro* using band shift assays. Scm3p-CEN binding *in vivo* requires Ndc10p but not Ndc80p or Spc25p, suggesting that Scm3p is downstream of the CBF3 complex but upstream of Ndc80p complex. Intriguingly, Scm3p also binds to microtubules at micromolar affinities *in vitro*, via a short $NH_2$-terminal domain that is dispensible for Ndc10p binding. Thus, Scm3p may function as a microtubule-binding protein that associates directly with CBF3, a hypothesis that is currently under investigation.

1626

**Counting the Number of Ndc80 and Dam1-DASH1 Protein Complexes at Budding Yeast Kinetochores by Quantitative Fluorescence Microscopy**

A. Joglekar, K. Bloom, E. D. Salmon; Biology, U. of N. Carolina, Chapel Hill, NC

In eukaryotes, the Ndc80 and Dam1-DASH complexes of kinetochore proteins are critical for anchoring spindle microtubules to the centromeres. As in higher eukaryotes including human cells, sister chromosomes in budding yeast get aligned at the spindle equator at metaphase, and segregate to the poles in anaphase by movement generated by kinetochores attached to the plus ends of spindle microtubules. Unlike higher eukaryotic

kinetochores which have multiple attached microtubules, each kinetochore in budding yeast is persistently attached to only one microtubule. This makes budding yeast ideal for extending the structural study of the microtubule-centromere connection to the molecular level. In budding yeast, the Ndc80 complex is proposed to link centromeres to microtubule plus ends by binding to a microtubule-associated ring of Dam1-DASH subunits. To measure the number protein molecules/kinetochore, we used digital imaging and analysis methods to quantify fluorescence signal at clusters of 16 kinetochores in anaphase-telophase cells expressing GFP-fusion protein from the native promoter. As a reference, we used Cse4p-GFP. Cse4p is a modified histone at the centromere, and the number of Cse4p molecules per kinetochore is thought to be either 2 or 4. The ratio of GFP-fusion protein fluorescence to that of Cse4p-GFP at kinetochores was ~ 3.5 for Nuf2p-GFP, ~ 3.5 for Ndc80p-GFP, thus confirming the stoichiometry measured by biochemical methods. The ratio was found to be ~ 6 for Ask1p-GFP, one of 10 proteins in the Dam1-DASH complex. These results indicate that anchorage of kinetochores to microtubule plus ends for chromosome segregation involves only 7-14 Ndc80 complexes and 12-24 Dam1-DASH complexes per microtubule attachment site. The same method may be extended for counting the number of other kinetochore complexes such as Ctf19, Mtw1, and Ndc10. Supported by NIH GM24364.

1627

**Regulation of Individual Kinetochore Microtubule Dynamics by Kinetochore Proteins in Budding Yeast**

J. F. Dorn,[1] K. Jaqaman,[1] G. S. Jelson,[2] J. D. Tytell,[2] C. Gonzalez,[2] P. K. Sorger,[2] G. Danuser[1]; [1]Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Biology, MIT, Cambridge, MA

Even in systems as simple as the budding yeast *S. cerevisiae*, the kinetochore consists of over 60 different proteins. The function of these proteins in assembly and maintenance of the kinetochore, and in cell cycle signaling is fairly well understood. However, their function in the mechano-chemical regulation of kinetochore microtubule dynamics is largely unknown, partly because mutations rarely result in strong dynamic phenotypes. We have established methods to probe the effect of kinetochore protein mutations on the dynamics of individual kinetochore microtubules, and to distinguish between phenotypes with unprecedented sensitivity. We record three-dimensional time-lapse movies of *S. cerevisiae* spindles that are fluorescently tagged at the spindle poles and at a centromere-proximal chromosomal region, and we recover microtubule length trajectories with super-resolution image analysis. Describing these trajectories with a combination of standard descriptors of microtubule dynamics and ARMA models allows us to statistically detect minute differences in kinetochore microtubule dynamics between wild type and mutant strains. For example, the temperature sensitive mutant *dam1-1* has been suggested to be unable to make strong attachments between kinetochores and microtubules. Indeed we find that in G1, in 20% of *dam1-1* cells microtubules detach from kinetochores. Our analysis is capable of distinguishing two more phenotypes among the cells with attachment, however: one half of these cells exhibits significantly perturbed regulation of microtubule growth and shrinkage, while in the other half the kinetochore seems to have completely lost the ability to regulate switches from microtubule growth to shrinkage. Such detailed readouts from a systematic screen of kinetochore proteins will allow us to elucidate the mechano-chemical role of kinetochore proteins.

1628

**The Kinetochore Ring Complex Moves Processively on Depolymerizing Microtubule Ends**

S. Westermann,[1] A. Avila-Sakar,[2] H. Wang,[2] D. G. Drubin,[1] E. Nogales,[3,1] G. Barnes[1]; [1]Molecular and Cell Biology, UC Berkeley, Berkeley, CA, [2]Lawrence Berkeley National Laboratory, Berkeley, CA, [3]Howard Hughes Medical Institute and Lawrence Berkeley National Laboratory, Berkeley, CA

The molecular mechanism by which the depolymerization of kinetochore microtubules drives the movement of chromosomes to poles during anaphase of mitosis has long remained elusive. In budding yeast the essential 10-protein Dam1 complex is the major microtubule-binding component of the kinetochore and the purified complex oligomerizes into a 50 nm ring around the microtubule. Here, we have developed a microscopy assay to directly observe the interaction of the complex with dynamic microtubules in vitro. Using live two -color fluorescence microscopy we show that Alexa labeled Dam1 ring complex moves processively at the ends of depolymerizing microtubules, where it slides for several micrometers without detaching from the lattice. The sliding mechanism is independent of the mode of microtubule disassembly as it is observed with both dilution and XMCAK induced depolymerization. While individual Dam1 rings slide effectively, a dense assembly of rings slows down or stalls microtubule depolymerization. On stable microtubules, Dam1 rings show undirected one-dimensional diffusion. To understand the structural basis for this type of microtubule based motility, we have examined electron micrographs of microtubule ends decorated with Dam1 ring complex. Image analysis of these “end-on” views reveals a 16-fold rotational symmetry of the complex with no apparent correlation to the microtubule lattice. This arrangement could allow the peeling protofilaments of depolymerizing microtubules to move the ring with a power-stroke mechanism. In conclusion we provide direct evidence that the Dam1 ring complex acts as a coupling device that can translate the force generated by microtubule depolymerization into movement along the lattice to drive chromosome segregation. The dynamic plus-end of the microtubule and the ring complex form a molecular machine that converts the chemical energy of GTP hydrolysis into mechanical work to generate directed movement independent of motor proteins.

1629

**Bir1p Links CBF3 to Passenger Proteins and Anaphase Progression**

S. Thomas, S. Hansen, A. Gillis, K. Kaplan; MCB, University of California, Davis, CA

The accurate segregation of chromosomes requires that anaphase is coordinated with the onset cytokinesis. In metazoans, kinetochore proteins called passenger proteins have been proposed to coordinate these events as they act at kinetochores and are required for inducing cytokinesis. In *Saccharomyces cerevisiae*, sequence homology suggests that Ipl1p, Sli15p and Bir1p are homologues of the metazoan passenger proteins, Aurora B, INCENP and Survivin, respectively. In addition, these proteins transit from kinetochores to interpolar microtubules in anaphase. Recent findings from our lab suggest that the inner kinetochore complex CBF3 works together with these passenger proteins to regulate the progression of anaphase and septin function necessary for proper cytokinesis. The CBF3 subunit Ndc10p interacts with Bir1p and mutations in CBF3 or BIR1 affect septins and cytokinesis. In contrast, mutations in IPL1 or SLI15 do not exhibit septin defects suggesting that these proteins regulate other aspects of anaphase. These results lead us to hypothesize that multiple passenger protein complexes form and regulate specific aspects of mitosis. To test this hypothesis, we have examined the interactions between CBF3 and passenger proteins using yeast two hybrid and *in vitro* biochemistry. Our

findings are consistent with Bir1p mediating the formation of distinct passenger complexes. Consistent with there being multiple Bir1p-passenger complexes, mutations in BIR1 compromise distinct aspects of mitosis causing aberrant chromosome segregation as well as cytokinetic defects. To further elucidate the mitotic functions of Bir1p complexes, we have created BIR1 mutants that specifically perturb its interaction with each passenger protein. This analysis will address the contribution of Bir1p complexes to both chromosome segregation and anaphase progression.

1630

**Identification of the Human Centromere Complex Proximal to the CENP-A Nucleosome**

D. R. Foltz,[1,2] B. E. Black,[1,2] A. O. Bailey,[3] J. R. Yates,[3] D. W. Cleveland[1,2]; [1]Ludwig Institute for Cancer Research, La Jolla, CA, [2]Department of Cellular and Molecular Medicine, UCSD, La Jolla, CA, [3]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

The faithful segregation of sister chromatids requires that each chromosome contain a single active centromere, the site of kinetochore assembly. The histone H3 variant CENP-A, in conjunction with the other core histones (H2A/H2B/H4), forms a centromere-specific nucleosome that plays an integral role in centromere function and may act as part of an epigenetic mark of the centromere and its associated kinetochore. To identify how the CENP-A nucleosome nucleates assembly of a functional kinetochore, an affinity purification approach has been used to purify CENP-A nucleosomes. Parallel purifications of histone H3.1 nucleosomes were also conducted in order to cull out factors also found in bulk chromatin. Several novel human centromere components were identified in each complex using mass spectrometry. Three of the factors associated with CENP-A nucleosomes, named CENP-K, CENP-L and CENP-M, were shown to be resident components of centromeres. Suppression of CENP-M by siRNA results in a mitotic phenotype that is consistent with an inability of chromosomes to make proper microtubule attachments. Subsequent affinity purifications of epitope tagged CENP-K, L, and M were conducted from stable cell lines each individually expressing one of the newly identified components. In each case purification of the tagged construct (CENP-K, L, or M) co-purified the other two proteins, demonstrating that CENP-K, L, and M exist within a common protein complex. Selective association of this complex with CENP-A nucleosomes derived from functional centromeres identifies these new centromere/kinetochore components as the most DNA proximal elements of the centromeric nucleosome.

1631

**Functional Replacement of CENP-A at the Centromere by a Chimeric Histone H3 Containing the CENP-A Targeting Domain (CATD)**

B. E. Black, L. E. T. Jansen, P. S. Maddox, J. V. Shah, D. R. Foltz, A. Desai, D. W. Cleveland; Ludwig Institute for Cancer Research, UCSD, La Jolla, CA

In humans, as well as in the overwhelming majority of all eukaryotic species, the chromosomal location of the centromere is not defined by a particular DNA sequence. Rather, all active centromeres are marked by the presence of a histone H3 variant, called CENP-A. While studying the features of CENP-A that are essential for its role in epigenetically marking and maintaining the location of the centromere we identified a portion of the histone fold domain consisting of the loop 1 and α2 helix of CENP-A that is sufficient to convert canonical H3 into a centromeric histone both in terms of its structural properties and sub-chromosomal targeting (Black *et al*., 2004, ***Nature*** 430:578-582). Here we have examined the degree to which this chimeric protein, $H3^{CATD}$, can replace CENP-A function. Using a plasmid-based sequential RNAi approach in HeLa cells, we have introduced a cocktail of selectable constructs against multiple CENP-A target sequences in order to deplete the endogenous protein. Stable expression of $H3^{CATD}$ rescues the long-term depletion of CENP-A that is lethal to control cells in colony outgrowth assays. Multiple clonal lines were isolated exhibiting both continued expression of $H3^{CATD}$ and an RNAi-directed reduction of endogenous CENP-A protein levels. These *rescued* cells have a normal cell cycle, arrest at metaphase upon treatment with spindle poison, recruit normal levels of centromere/kinetochore proteins, and undergo a normal mitotic division as observed by live cell microscopy. Subsequent inhibition of $H3^{CATD}$ expression was lethal, indicating that $H3^{CATD}$ provides an essential function when CENP-A is depleted. Thus, the major if not sole role of CENP-A is to provide a unique nucleosome structure that distinguishes the centromere from the rest of the chromosome.

1632

**Identification of Factors Involved in Centromeric Chromatin Formation**

C. M. Betts,[1] S. Erhardt,[2] B. Mellone,[2] G. Karpen,[2] A. F. Straight[1]; [1]Biochemistry, Stanford Medical School, Stanford, CA, [2]Molecular and Cellular Biology, UC Berkeley, Berkeley, CA

Identification of factors involved in centromeric chromatin formation. Accurate chromosome segregation in mitosis requires attachment of chromosomes to the microtubule spindle through the kinetochore. During mitosis, kinetochores are assembled on the specialized chromatin of the centromere. Centromeric chromatin is characterized by a core region containing nucleosomes in which Cenp-A, the centromeric histone H3 variant, replaces histone H3. The centromere is embedded in large regions of heterochromatin characterized by repressive histone modifications and the localization of the heterochromatin marker, heterochromatin protein 1 (HP1). The pathways that control the incorporation of Cenp-A into chromatin and the assembly of the centromere are unknown. In an effort to identify factors responsible for Cenp-A incorporation into chromatin and pericentric heterochromatin formation, we performed a whole-genome RNAi screen in *Drosophila melanogaster* cultured cells. *Drosophila* tissue culture cells were treated with double stranded RNAi corresponding to each gene in the *Drosophila* genome, fixed and stained for DNA, CID (the *Drosophila* Cenp-A homologue), and HP1 and then imaged using high throughput automated microscopy. We have developed computational methods to automate image segmentation and analysis; this has allowed us to take quantitative measurements at the single cell level and rapidly identify potential CID and HP1 interacting factors. We have identified three classes of genes: those that are specifically required for the localization of Cenp-A to the centromere, those that are necessary for proper localization of HP1 and those that modify both processes. This screen should allow us to comprehensively analyze the process of centromere and heterochromatin formation in eukaryotes.

1633

**Phosphorylation of Hec1/hNdc80 is Important in Ndc80 Complex Function**

S. A. Miller,[1] M. L. McCleland,[2] P. T. Stukenberg[1]; [1]Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, [2]Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA

In mitosis, a large, proteinaceous structure called the kinetochore, assembles on centromeric heterochromatin and is responsible for the faithful

transmission of a single complement of chromosomes to each daughter cell. The kinetochores of sister chromatids must be attached to opposite spindle poles before they can be properly segregated. Improperly attached kinetochores generate an “anaphase wait” signal that halts the cell cycle until all sister chromatids are aligned on the metaphase plate with proper bipolar attachments. The Ndc80 complex has been shown to play a role in kinetochore structure, microtubule-kinetochore attachment, and spindle checkpoint signaling. Although it is involved in all of the functions of the kinetochore, the mechanism and regulation of the Ndc80 complex are poorly understood. We systematically screened kinetochore proteins for the ability to be substrates for post-translational modification and found that both Hec1/hNdc80 and xNdc80 can be phosphorylated by Aurora B in vitro. It is believed that the mitotic kinase Aurora B is essential for faithful chromosome segregation by releasing improperly attached microtubules from kinetochores. We are currently mapping the phosphorylation site(s) as well as investigating the effect of this modification on Ndc80 complex function.

1634

**Examination of the Kinetochores Microtubule Nucleation and Binding Activities**

M. J. Emanuele,[1] M. J. Kallio,[2] G. J. Gorbsky,[3] T. Stukenberg[1]; [1]Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, [2]Turku Centre for Biotechnology, Turku, Finland, [3]Molecular, Cell and Developmental Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK

The vertebrate kinetochore assembles on centromeres from over 50 proteins into a structure which mediates microtubule attachment and chromosome movement during mitosis. Prior to the identification of its numerous components, the kinetochores biochemical activities with respect to microtubules were defined in classic studies from Mitchison and Kirschner (1985). These in vitro activities included the ability of kinetochores to both bind and nucleate microtubules. An important unanswered question is which kinetochore proteins mediate these activities. Using function blocking antibodies and pharmacological inhibitors we have begun to address the in vitro functions of numerous kinetochore proteins. Inhibition of the outer plate protein Ndc80 has a clear effect on the kinetochores ability to bind microtubules. This data, combined with the observed phenotypes from tissue culture cell injections, further confirms a critical role for Ndc80 in microtubule binding. In addition, antibodies raised against Zwilch, a member of the Rod Complex, have a similar effect on the kinetochores ability to bind microtubules. These results implicate the Rod complex as having a role in microtubule binding at the kinetochore. The continued utilization of this in vitro system will provide us with a better understanding of the biochemical activities of many known kinetochore proteins.

1635

**Lagging without Bub3: New Role for the Checkpoint Protein Bub3 at the Kinetochore-Microtubule Interface**

E. Logarinho, T. Resende, H. Bousbaa; Grupo de Biologia Celular e Molecular, CESPU, Instituto Superior de Ciencias da Saude-Norte, Gandra PRD, Portugal

The genomic stability of all organisms depends on the correct segregation of chromosomes during cell division. The accuracy of this process is monitored by the spindle checkpoint, a signal transduction system comprised of conserved Mad, Bub and other proteins, which act to induce a prometaphase arrest until proper bipolar attachment is achieved. Among the checkpoint proteins, Bub3 has remained the one most poorly studied. Therefore, we further investigated the functions of the human checkpoint protein Bub3 at the kinetochore-microtubule interface. We show that RNAi depletion of Bub3 causes a prometaphase delay, indicative of a checkpoint response. Interestingly, and in contrast to other Bub knockdown phenotypes, chromosomes in Bub3-depleted cells are able to congress and even organize a metaphase plate that, however, exhibits a few permanently misaligned chromosomes close to the plate. We show that kinetochore-microtubule attachments are unstable in the absence of Bub3. Also a significant fraction of Bub3-depleted cells enter anaphase with lagging chromosomes. Furthermore, we show that Bub3 is required for kinetochore localization of Mad2 and Cdc20, the checkpoint effectors that halt anaphase, and we relate it to the high number of anaphases with lagging chromosomes observed for Bub3-depleted cells. However, persistent low levels of Mad2 can also contribute to the prometaphase delay found for the Bub3 knockdown, as cells exit mitosis prematurely upon Mad2 depletion. We propose Bub3 as a core component of a kinetochore catalytic platform which directly links the establishment of proper kinetochore-microtubule attachments to the activation of the anaphase inhibitory checkpoint complex.

1636

**Structural and Functional Analysis of Human Zw10 Interacting Protein, hZwint**

L. J. Vos, G. K. T. Chan; Oncology, University of Alberta, Edmonton, AB, Canada

The human Zw10 interacting protein, hZwint, is a novel kinetochore protein originally discovered by a yeast 2-hybrid screen for interactors of Zw10 (Starr *et al*. 2000). hZw10 and hROD form a complex which recruits dynein/dynactin to the kinetochore and it is an essential component of the mitotic checkpoint (Chan *et al.* 2000). hZwint localizes to the kinetochore in prophase, prior to hZw10 localization, and as such that hZwint might be a scaffold protein to which hZw10 binds. We are examining the domain(s) that are necessary for hZwint kinetochore localization and hZw10 binding. We are using two approaches for mapping the domains of hZwint. We have created a library of 23 hZwint mutants with 5 amino acids randomly insertion. We have analyzed these mutants to determine the domain(s) that are important for hZwint kinetochore localization. Based on these results, we have further produced 17 mutants by site-directed mutagenesis. By transiently transfecting HeLa cells with these mutants in a GFP-hZwint vector, it was determined that the N-terminal region is crucial for kinetochore localization. The Zw10 interacting domain is being mapped by employing the mutants in yeast 2-hybrid, co-immunoprecipitation and fluorescence resonance energy transfer (FRET) analysis. With the structural mapping of hZwint, I am hoping to analyze its function by designing dominant negative mutants that bind kinetochores but cannot interact with Zw10.

1637

**Three-dimensional Protein Domains at Centromeres**

O. J. Marshall,[1,2] A. T. Marshall,[3] K. H. A. Choo[1,2]; [1]Chromosome Research, Murdoch Childrens Research Institute, Melbourne, Australia, [2]Department of Paediatrics, The University of Melbourne, Melbourne, Australia, [3]Department of Zoology, La Trobe University, Melbourne, Australia

Throughout all eukaryotes, the centromere is the fundamental structure that controls the correct segregation of chromosomes during cell division. Although many of the major centromere proteins are known and have been characterised via immuno-fluorescence, few have been studied in detail using immuno-electron microscopy, none have been mapped across the three-dimensional kinetochore structure, and no model has been produced that relates the extant chromatin immunoprecipitation data with the physical evidence of immuno-electron microscopy. CENP-A is a modified histone protein that replaces histone H3 in centromeric chromatin at the kinetochore. Its presence is essential for the correct loading of many other centromere proteins and thus for the formation of a functional kinetochore. Here we describe for the first time the detailed domain structure of CENP-A, defined in relation to the kinetochore and the primary constriction. Using immuno-electron microscopy a three-dimensional model of the constriction, the kinetochore and the region of chromatin bound by the protein can be constructed via serial sections. We demonstrate that CENP-A occupies a domain that stretches across the kinetochore in a narrow band, occupying only a fraction of the height and depth of the constriction. Combining this in vivo structure with the linear domain maps generated by chromatin immuno-precipitation, we suggest a new model for chromatin folding at the centromere.

1638

**Functional Analysis of hMis12-Associated Kinetochore Proteins in Human Cells**

S. L. Kline-Smith, I. M. Cheeseman, A. Desai; Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA

Accurate chromosome segregation relies on precisely regulated interactions between kinetochores of mitotic chromosomes and microtubules of the spindle. In previous work, we identified a network of 10 interacting kinetochore proteins conserved between C. elegans and human cells. This network includes the HEC1/Ndc80 complex, Mis12 and KNL-1. Here, we present functional analysis of three novel human kinetochore proteins, DC31, PMF1 and Q9H410, which were identified utilizing tandem affinity purification of hMis12. Like hMis12, DC31, PMF1 and Q9H410 localized to kinetochores coincident with the inner kinetochore protein CENP-A throughout mitosis. RNAi-induced depletion of DC31, PMF1 or Q9H410 suggests that these proteins are not required for kinetochore targeting of CENP-A. In addition, DC31, PMF1, Q9H410 and hMis12 exhibited inter-dependence for their kinetochore localization, suggesting that they may function as a subcomplex within the kinetochore. Knockdown of DC31, PMF1 or Q9H410 resulted in cells with severe defects in chromosome alignment, although spindle formation appeared to be normal. Many depleted cells remained in mitosis for more than 10 hours as judged by analysis of fluorescently labeled chromosomes in living cells. Despite this extended delay, chromosomes were never able to fully congress to the metaphase plate, and many cells contained abnormally positioned chromosomes located outside the region between spindle poles. Live analysis of fluorescently labeled kinetochores suggests that chromosomes in depleted cells could attach to microtubules, as kinetochore oscillations were observed. Unlike kinetochore fibers in control cells, many k-fibers in depleted cells depolymerized upon cold treatment. This instability of k-fibers is likely due to a reduction in kinetochore-localized HEC1/Ndc80, which was observed in depleted cells. These results provide evidence that DC31, PMF1 and Q9H410 function with hMis12 at the inner kinetochore and are critical for proper chromosome alignment and progression through the cell cycle in human cells.

1639

**The Role of the P97-Ufd1-Npl4 Complex in Chromosome Congression During Mitosis**

Q. Vong, Y. Zheng; Carnegie Institute, Baltimore, MD

The AAA-ATPase Cdc48/p97 and its adapters Ufd1-Npl4 (U-N) form a complex (p97-U-N) that has a well-established role in regulating interphase membrane functions. Some of the membrane functions of the p97-U-N complex have been linked to the ubiquitin and proteasome system. Recently, we showed that the p97-U-N complex also has a role in mitosis by regulating spindle disassembly in a separate pathway from the mitotic exit network. We have now further studied the function of this complex in mitosis and found that it has an earlier role in mitosis. RNAi-mediated down-regulation of Ufd1 significantly prolonged the time HeLa cells spend in prometaphase and metaphase. Time-lapse imaging revealed that many mitotic cells treated by Ufd1 siRNA have difficulty in achieving chromosome congression. Interestingly, after a delay, many cells proceeded into anaphase without chromosome congression. This led to failure in cytokinesis. Consistent with our previous finding, Ufd1 down-regulation did not block spindle assembly. However, we observed an increase in multipolar spindles in Ufd1 siRNA-treated cells as compared to control siRNA-treated cells. We are in the process of identifying the down-stream target(s) of p97-U-N that regulates chromosome congression.

1640

**Cenp-F and Vertebrate Mitosis**

A. Salmela,[1,2] A. Varis,[3,1] M. J. Kallio[1,3]; [1]Cancer Biology and Cell Screening, VTT Medical Biotechnology, Turku, Finland, [2]Turku Graduate School of Biomedical Sciences, University of Turku, Turku, Finland, [3]Centre for Biotechnology, University of Turku, Turku, Finland

The spindle assembly checkpoint (SAC) safeguards genomic stability by preventing sister chromatid separation in presence of a misaligned or unattached chromosome. Kinetochores of chromosomes are composed of stable structural proteins and transiently associating molecules many of which are SAC molecules that promote or inhibit mitotic progression in a microtubule attachment and kinetochore tension dependent manner. We are investigating Cenp-F, a large 350 kD coiled-coil protein that transiently associates with mitotic kinetochores. The protein has been proposed to have multiple functions in cell proliferation but its mechanism of action is poorly understood. To study Cenp-F function we utilized two different techniques. First, by introducing Cenp-F siRNA into human tissue culture we demonstrate a role for the protein in chromosome alignment and SAC signalling. Most cells in a Cenp-F siRNA treated population have one or more chromosomes unaligned that causes a pseudometaphase arrest. Many of these cells however, undergo anaphase and exit M phase despite the presence of misaligned chromosomes. BubR1 is not efficiently retained at the unaligned kinetochores in Cenp-F depleted cells. In another approach, we microinjected antibodies against Cenp-F into human tissue culture cells at different phases of mitosis. The average time the anti-Cenp-F antibody injected cells spend at metaphase is significantly longer compared to controls. Moreover, in some antibody injected cells one or more chromosomes fail to congress to the cell equator. Our results support previously proposed hypothesis that the few unaligned chromosomes of Cenp-F silenced cells are not sufficient to create SAC signals to inhibit onset of anaphase. This could be due to inability to retain BubR1 at these kinetochores. However, when the SAC is hyperactivated by induction of spindle damage and the kinetochores of all chromosomes contribute to creation of the “wait anaphase” signal, the cells accumulate at mitosis.

1641

**Hec1 Regulates Kinetochore-Microtubule Dynamics Through its N-terminal Globular Domain**

J. G. DeLuca, E. D. Salmon; Biology, UNC-Chapel Hill, Chapel Hill, NC

Chromosome movements during mitosis are dependent on forces that are generated in the kinetochore outer plate by attached microtubules. The Ndc80 complex, comprised of Ndc80 (Hec1), Nuf2, Spc24, and Spc25, is essential for kinetochore microtubule formation and metaphase chromosome alignment. To further understand the role of Hec1 in chromosome motility, we injected monoclonal antibodies to Hec1 into mitotic PtK1 cells. Epitope mapping revealed antibody binding at the N-terminal globular domain of Hec1, away from the protein regions involved in dimerization with Nuf2 and tetramerization with Spc24 and Spc25, and putatively away from kinetochore-targeting regions. In PtK1 cells, injected Hec1 antibodies localized to kinetochores, consistent with Hec1's known localization to the kinetochore outer plate. Injected cells exhibited poor chromosome alignment and underwent anaphase with unaligned chromosomes. Interestingly, all kinetochores of injected cells were able to form robust kinetochore microtubules, but there was a significant increase in attachment errors, including syntelic attachment and merotelic attachment. Live cell imaging revealed that sister kinetochores in injected cells were hyper-stretched, and no kinetochore oscillations were observed, suggesting that Hec1 plays a role in regulating microtubule dynamics at kinetochores. Using a photo-activatable GFP-Tubulin-expressing PtK1 cell line, we observed a decrease in microtubule turnover at kinetochores in injected cells. In addition, kinetochore microtubules in EB1-GFP expressing cells were unable to persistently polymerize after injection of Hec1 antibodies. Our results suggest that antibodies to the N-terminus of Hec1 block kinetochore microtubules from growing or shortening, and thereby prevent chromosome movement and error correction. These findings reveal a key role for the N-terminal globular region of Hec1 in controlling the dynamic behavior of kinetochore microtubules. Supported by NIH GM24364 to EDS and NIH GM66588 to JGD.

1642

**Arrangement of Fibers and Microtubules in the Mammalian Kinetochore**

Y. Dong, K. Vendenbeldt, X. Meng, B. F. McEwen; Wadsworth Center, New York State Dept Health, Albany, NY

The dynamic interaction between microtubules and kinetochores during mitosis is essential for chromosome attachment to the mitotic spindle, chromosome movement, and activation of mitotic checkpoint. Recent studies have begun to define the molecular composition of the kinetochore with more than 60 molecular components identified in *S. cerevisiae*. Several homologues of these proteins have been found in vertebrates. Kinetochore proteins form at least 14 different complexes that assemble hierarchically into an inner core, a linker domain, and an outer domain. This arrangement reflects the classical structural model for the vertebrate kinetochore, which consists of an inner plate, an outer plate, and corona material. The heterotetrameric Ndc80 complex is located within the kinetochore outer plate, where it functions to organize the microtubule-kinetochore interface. Recombinant human Ndc80 complex forms an elongated shape that is 40-45 nm long and is predicted to align perpendicularly to the outer plate. In this study, we use electron tomography of $PtK_1$ cells prepared by high-pressure freezing and freeze substitution to obtain a high resolution structural map of the kinetochore outer plate. Our results show that the outer plate of the unbound Ptk1 kinetochore is composed of a network of cross-linked fibers which are roughly parallel to the outer plate surface. The average length of these fibers is close to the length of Ndc80 coiled-coil structure. This suggests that Ndc80 is oriented parallel rather than perpendicular to the outer plate surface. Upon microtubule binding, the fibrous arrangement becomes less organized and tends to form a cage-like structure around the microtubule plus ends. In addition, there are irregular attachments directly to the microtubule plus ends that likely influence microtubule dynamics. Supported by NSF grant MCB0110821 and NIH grants R01GN6627001, and P41RR01219.

1643

**Sgt1p interacts with Hsp82p and Sti1p to Form a Chaperone Complex**

M. G. Catlett, K. B. Kaplan; Molecular and Cellular Biology, University of California, Davis, Davis, CA

Sgt1p is an essential protein found in all eukaryotes. In budding yeast, Sgt1p is required for formation of the CBF3 core kinetochore complex as well as additional roles including progression beyond G1. While the biochemical function of Sgt1p remains unknown, Sgt1p contains two domains that suggest it could be an Hsp90 co-chaperone: an N-terminal tetracopeptide repeat (TPR) and a C-terminal domain with homology to the known co-chaperone p23/Sba1p. Hsp90p co-chaperones bind to Hsp90p and affect the substrate specificity of the chaperone. Recent results from numerous organisms have shown that Sgt1p and Hsp90p interact in vivo, suggesting that Sgt1p may be a bona fide co-chaperone. However, in contrast to characterized co-chaperones, only a small percentage of Sgt1p associates with Hsp90p. To better understand how Sgt1p influences Hsp90p function, we have used recombinant proteins to reconstitute the interactions between Sgt1p, Hsp82p (one of two yeast HSP90 genes) and other co-chaperones. We find that Sgt1p binds directly to Hsp82p and that this interaction is sensitive to the nucleotide bound state of Hsp82p. Further, we find that Sgt1p can bind Hsp82p in the presence of other co-chaperones, including Sti1p (Hop in human cells). Genetic and biochemical approaches are consistent with the possibility that Sgt1p, Sti1p and Hsp82p form a functional complex. As co-chaperones often interrupt the ATPase cycle of Hsp90p, we have examined the effect of Sgt1p on the ATPase rate of purified Hsp82p and see no evidence that Sgt1p can inhibit the chaperone's ATP turn-over rate. To address how this novel chaperone complex interacts with bona fide clients, we propose to reconstitute the steps necessary to assembly the CBF3 complex from purified components.

1644

**Caspase-Independent Mitotic Apoptosis (CIMA): A Novel Type of Caspase-Independent Cell Death That Occurs during Mitosis in BUB1-Deficient Cells**

Y. NIikura, K. Kitagawa; Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN

The spindle checkpoint protects cells from aneuploidy by monitoring the status of the kinetochore-microtubule attachment. When cells have defects in this mitotic checkpoint pathway and in kinetochore-microtubule attachment, substantial aneuploidy is thought to result. We report here a novel type of caspase-independent apoptosis that occurs during mitosis_CIMA (caspase-independent mitotic apoptosis). Simultaneous depletion of BUB1, but not MAD2, and treatment with nocodazole (a microtubule-depolymerizing drug), paclitaxel (Taxol, a microtubule-stabilizing drug), or 17-AAG (17-allylaminogeldanamycin, an HSP90 inhibitor) induced DNA fragmentation in cells during early mitosis. The common defects caused by the microtubule inhibitors and 17-AAG are defects in kinetochore-microtubule attachment. Therefore, we conclude that CIMA occurs when the

kinetochore-microtubule attachment is altered and BUB1 function is disrupted. This novel mitotic cell death appeared to be independent of caspase activation. We found that AIF (apoptosis-inducing factor) and EndoG (endonuclease G), which are effectors of caspase-independent apoptosis, are released from mitochondria during the activation of CIMA, and that DNA fragmentation is dependent on EndoG. Our results suggest that BUB1 is a critical factor in regulating CIMA, and we propose that CIMA protects cells from aneuploidy by inducing the death of cells prone to substantial chromosome missegregation.

## Pre-College and College Science Education (1645-1676)

1645

### Understanding of Mitosis and Meiosis and Genetic Information Among Higher Secondary Students of Northeast India and the Implications in Genetics Education

A. Chattopadhyay; Centre for Science Education, North Eastern Hill University, Shillong, India

Almost hundred years after coining of the terms “genetics” and “gene” the science of genetics has become increasingly molecular particularly after the work of Watson and Crick (1953). By the end of the twentieth century, reports of animal cloning and recent completion of the Human Genome Project, as well as techniques developed for DNA fingerprinting, gene therapy and others, raised important ethical and social issues, about applications of such technologies. To understand these issues, appropriate genetics education is needed in schools. A good foundation in genetics also requires knowledge and understanding of topics such as structure and function of cells, cell division, and reproduction. Studies at the international level report poor understanding by students of genetics and genetic technologies, with widespread misconceptions at various levels. Similar studies were nearly absent in India. In this study, I examine Indian higher secondary students’ (289 students; 16-18 yrs; 3 colleges) understanding of mitosis, meiosis, genetic information related to cells using written questionnaire. It was observed that most of the students had a fragmented knowledge, incomplete and inconsistent in nature without a coherent view of the cells, chromosomes and genetic information within the cells [Chattopadhyay (2005), Cell Biology Education, 4, spring, 97 - 104]. These misconceptions were rooted in their lack of understanding of mitosis and meiosis. Only 15% - 40% students ticked the correct choice and gave a scientifically valid reason. Although preliminary in nature, the results provide cause for concern over the status of genetics education in India. The possible reasons for such misunderstanding and the probable steps to overcome such problems will be discussed in the paper.

1646

### Community Building to Promote Careers in Biomedical Science

C. H. Evans, M. I. Klopf, A. L. French; Department of Human Science, Georgetown University, Washington, DC

We have developed a rural underserved community partner education model to stimulate interest of high school students in biomedical science by working with students, their school, and community mentors. The goals are to 1) increase engagement of individuals from rural areas in the intellectual and work life of biomedical scientists and health care professionals and 2) test the usefulness of a technologically-driven education model to higher education institutions in drawing rural communities into the intellectually and culturally rich life of institutes of higher learning. 70 high school junior and senior students and adults (52 youth and 18 adults) from the Oglala Lakota Sioux American Indian Pine Ridge Reservation in SD (20 participants), the African-American and Cajun Assumption Parish, LA community (36 participants) and Mexican-American migrant farm worker communities in Florida and Texas (14 participants) traveled to Georgetown University during 2003-5 for 3-week summer science institutes. The on-campus residential experiences featured educational skill evaluation and science classroom and hands on laboratory instruction by Georgetown University faculty, students, and invited experts, as well as career exploration activities. Continuing biomedical science education throughout the year is provided through a biological virtual laboratory that offers students innovative exercises to build qualitative and quantitative science critical thinking and analysis, and communications skills. Validating the effectiveness of this biomedical science education model for increasing individuals in the biomedical and health care related pipeline is being pursued by measuring the success of student participants in entering college and evaluating the biological virtual laboratory as a useful tool for distance interaction, learning and quantitative assessment. Supported by a NIH NCRR Science Education Partnership Award.

1468

### Community Building to Promote Careers in Biomedical Science

C. H. Evans, M. I. Klopf, A. L. French; Department of Human Science, Georgetown University, Washington, DC

We have developed a rural underserved community partner education model to stimulate interest of high school students in biomedical science by working with students, their school, and community mentors. The goals are to 1) increase engagement of individuals from rural areas in the intellectual and work life of biomedical scientists and health care professionals and 2) test the usefulness of a technologically-driven education model to higher education institutions in drawing rural communities into the intellectually and culturally rich life of institutes of higher learning. 70 high school junior and senior students and adults (52 youth and 18 adults) from the Oglala Lakota Sioux American Indian Pine Ridge Reservation in SD (20 participants), the African-American and Cajun Assumption Parish, LA community (36 participants) and Mexican-American migrant farm worker communities in Florida and Texas (14 participants) traveled to Georgetown University during 2003-5 for 3-week summer science institutes. The on-campus residential experiences featured educational skill evaluation and science classroom and hands on laboratory instruction by Georgetown University faculty, students, and invited experts, as well as career exploration activities. Continuing biomedical science education throughout the year is provided through a biological virtual laboratory that offers students innovative exercises to build qualitative and quantitative science critical thinking and analysis, and communications skills. Validating the effectiveness of this biomedical science education model for increasing individuals in the biomedical and health care related pipeline is being pursued by measuring the success of student participants in entering college and evaluating the biological virtual laboratory as a useful tool for distance interaction, learning and quantitative assessment. Supported by a NIH NCRR Science Education Partnership Award.

1647

**The Microscope Imaging Station: Choosing Biomedically Relevant Samples for Public View**

K. R. Yu,[1] J. Ma,[2] C. Carlson[1]; [1]Life Sciences, Exploratorium, San Francisco, CA, [2]Visitor Research and Evaluation, Exploratorium, San Francisco, CA

The Exploratorium, an interactive museum of science, art, and human perception, has developed an interactive Microscope Imaging Station (MIS) for use by museum visitors. The MIS provides museum visitors with the unique opportunity to control research-grade microscopes to explore live, biomedically relevant samples. The imagery from these samples presents a source of educationally rich material. However, microscopic imagery can also serve be a significant source of visitor confusion and frustration, if not thoughtfully presented. One of the most critical choices in the development of the MIS was the presentation of live, biomedically relevant samples that were appropriate for public view. Through a series of evaluation studies with visitors and logistical evaluations in the museum laboratory, we have developed guidelines that are used to choose appropriate samples and associated educational messages. Here we discuss the conceptual hurdles involved in choosing samples, propose some criteria for the determination of appropriate educational specimens, and explore the need for a visual hook as well as personal or topical relevance when presenting biology to the museum-going public.

1648

**Classroom Exercise: Treating Bull Sperm with Caffeinated Drinks Induces Hyperactivation**

P. C. Jones,[1] S. Suarez[2]; [1]Science, Homer High School, Homer, NY, [2]Biomedical Sciences, Cornell University, Ithaca, NY

A laboratory exercise was developed to enable students to examine the effects of caffeinated drinks on sperm motility. Caffeine induces hyperactivation in mammalian sperm by raising intracellular $Ca^{2+}$. Hyperactivation is required for fertilization, particularly sperm penetration of the oocyte zona pellucida. On a microscope slide, hyperactivated sperm swim rapidly in circles because of an asymmetrical increase in flagellar beat amplitude. High school teachers can start the lab as an introduction to mammalian reproduction. Teachers can purchase bull semen frozen in egg yolk extender and packaged in 0.5 ml plastic artificial insemination straws. Students working in pairs can thaw semen by submerging a straw in a 37C water bath for 30 sec. The straw is then emptied into 2 ml of Carnation Instant Non-Fat Milk prepared with distilled water as per instructions on the package and brought to pH 8.3 using 1 molar NaOH. The students can observe the sperm under a microscope using a 40x objective to see their linear, progressive swimming movements. Students can then add instant coffee (dissolved in milk diluent and adjusted to pH 8.3 with 1 M NaOH) to sperm at a 2:5 dilution and observe them immediately. Approximately half of the sperm will be killed by the introduction of coffee; however, some surviving sperm will exhibit hyperactivation. As a control, students can treat sperm with decaffeinated coffee. Again, half will be killed; however, the surviving sperm will not exhibit hyperactivation. The experiment can be expanded to include tea and cola drinks. This exercise can be used to stimulate student discussion about fertilization, possible effects of caffeine on their bodies, and using proper experimental controls (caffeinated *vs* decaffeinated drinks). Development of the exercise was supported by a Research Experiences for Teachers supplement to NSF grant MCB-0421855.

1649

**Influencing Science Graduate Students' Professional Identities through K-12 Partnerships**

A. K. Busch, K. D. Tanner; Biology, San Francisco State University, San Francisco, CA

With the growth of scientist-teacher collaborations, scientists are increasingly placed in K-12 classrooms with little or no formal training in science education, pedagogy, or partnership. These collaborations are often viewed as service or outreach for participating graduate student scientists and typically occur outside formal graduate school experiences. Consequently, insights into science teaching and learning may not become integrated into scientists' identities as researchers and future undergraduate instructors. In 2004, the Science Education Partnership and Assessment Laboratory (SEPAL) at San Francisco State University (SFSU) extended previous research on the impact of partnerships on scientists (Tanner, 2000; Phillips, 2002) and began examining the impact of formally integrating K-12 partnership experiences into the training of graduate students. Specifically, the SEPAL GK-12 partnership program engages SFSU Master's science students in time-intensive, one-on-one partnerships with secondary science teachers, simultaneous with a seminar course in science education and pedagogy. The research reported here examines how these scientific trainees were influenced by this formalized science education aspect of their graduate experience. Through reflective essays and pre- and post-assessment responses, graduate students reported that this experience: 1) improved their ability to communicate their thesis research, 2) significantly influenced their teaching philosophy, 3) established for them a role for scientists in K-12 education, and 4) enabled them to articulate a new professional identity as a "scientist educator." These results extend previous findings and suggest that inclusion of formal training in science education into the graduate school experience enabled these graduate students to more fully integrate their K-12 partnership experiences into their identities as developing scientists. As such, formalizing partnership experiences as part of graduate training is a promising approach to generating young scientists that consider K-20+ science education reform integral to their professional lives. [NSF #DGE0337949]

1650

**Student Models of Cell Division Measure Ongoing Conceptual Gains and Challenges**

E. M. Stone; Berkeley High School, Berkeley, CA

Scientists build models to represent and explain their ideas, test alternate hypotheses, and brainstorm solutions to problems. Research in science education tells us that that not only is building models an essential part of the practice of science, but it can also be crucial for students in learning complex concepts. A computer-based high school curriculum called "Cell Reproduction & Human Diseases" was designed in the Web-based Inquiry Science Environment (WISE, http://wise.berkeley.edu) to promote cell division learning by guiding students to create models of related human diseases. Models were analyzed at three points in the unit- early, middle and late- with rubrics that determined students' conceptual understanding of the structure and function of cellular components required for cell division, and their ability to reason about causes of diseases. The rubrics were particularly useful for measuring student drawings, which are typically difficult to analyze objectively for evidence of learning. Data obtained from scoring with these rubrics showed that students progressively integrated their knowledge of cell division from the beginning to the end of the unit. Moreover, students developed a greater understanding of the nature of science by learning to evaluate their own models and to appreciate the purpose of models in general. This study demonstrated that adaptations to the curriculum after its first year resulted in increased

learning gains in the second year of its implementation. Additionally, analysis of student data demonstrated areas for which students continue to have difficulty in conceptual understanding, which suggests changes in the curriculum for a third year of implementation. Overall, this work identifies ways in which instructors may undertake ongoing formative assessment of their curricula and introduce iterative modifications that result in improved student learning.

1651

### A Novel Summer Program Designed to Educate and Train Students for a Career in Bioinformatics

J. Momand,[1] W. Johnston,[2] S. Sharp,[3] N. Warter-Perez,[4] B. Krilowicz[3]; [1]Chemistry and Biochemistry, California State University Los Angeles, Los Angeles, CA, [2]Biology, Pasadena City College, Pasadena, CA, [3]Biological Sciences, California State University Los Angeles, Los Angeles, CA, [4]Electrical Engineering, California State University Los Angeles, Los Angeles, CA

**Objective:** To design and implement a summer program for undergraduates and graduate students that teaches bioinformatics concepts, offers skills in professional development, and provides research opportunities in bioinformatics. **Methods:** The program, first implemented in Summer 2003, was divided into a three week didactic session followed by a seven week research internship. The didactic session addressed topics in bioinformatics, ethics, statistics, and computer programming. Professional development sessions helped students develop skills in oral presentation, milestone setting, and resume writing. Students were placed into bioinformatics research laboratories at five academic research institutions and four companies. At the end of the ten-week period students presented their research to instructors, research mentors and peers. **Results:** Combined assessments of "overall teaching ability" of seven instructors by participants (n=24) enrolled in programs of 2003 and 2004 indicated that 87-91% of participants ranked instructors "good" to "excellent" with 9-13% ranking them in the "fair" or "no opinion" category. Students' performances on culminating research presentations were ranked by principal investigators, research mentors, and peers using quantitative rubrics designed to assess specific aspects of final oral presentations. On a scale of 1 to 4, with 1 = not acceptable and 4 = excellent, mean overall student (n = 24 interns over 2 years) performance were rated 3.4 by principal investigators (n= 3-4), 3.1-3.3 by research mentors (n = 1-4), and 3.5-3.6 by peers (n= 12 for each year). **Conclusions:** Assessment data suggest that the program is offering high quality education and training. Out of 24 participants, 16 are continuing their education in a field related to bioinformatics, 4 are searching for employment, and 1 is employed outside the bioinformatics sector. The status of three participants is not known.

1652

### Total Immersion Biology: A Course That Engages First-Year Undergraduate Students in Cell Biology Research

C. A. Lindgren,[1] C. H. Sullivan,[1] D. E. Lopatto[2]; [1]Biology, Grinnell College, Grinnell, IA, [2]Psychology, Grinnell College, Grinnell, IA

To enhance the environment for collaborative student/faculty research, many departments offer a research methods course to students in their second or third year of undergraduate study. We offer such a course that emphasizes the process of science. The difference is that this course is the **first** course students take. Multiple sections of *Introduction to Biological Inquiry* are offered each semester. Each faculty member teaches a section focused on a unique biological question that is related to her or his research interests. However, all sections teach similar skills such as reading the scientific literature, defining a good question, designing experiments, learning the tools to carry out that experiment, analyzing and presenting data, and writing scientific papers. Instead of the more traditional three lecture classes and one three-hour lab per week, each section is taught in the "workshop" format where lecture, laboratory, and discussion are integrated. Students meet two or three times a week in larger blocks of time that usually total the same six hours per week. The course concludes with a poster session where students from all sections of the course present their major projects to one another. The students who continue in our curriculum to the next course in *Molecules, Cells, and Organisms* bring a variety of perspectives based on the section of *Introduction to Biological Inquiry* they took. On end of course evaluations, students indicated that they (1) had better insight into how biological knowledge was advanced, (2) were more confident in their ability to understand new scientific topics, (3) were more confident in their ability to critique scientific work and (4) improved their writing skills. Pre-test/post-test comparisons demonstrate significant improvement in the areas of factual information, data interpretation, and experimental design.

1653

### Freshman and Sophomore Research Workshops for Early Research Awareness

V. Navas,[1] M. Chaparro,[1] R. Montalvo,[1] N. Diffoot,[1] D. Ramirez,[2] R. Buxeda[1]; [1]Biology, University of Puerto Rico Mayaguez Campus, Mayaguez, PR, [2]Chemistry, University of Puerto Rico Mayaguez Campus, Mayaguez, PR

The Biology Department at the University of Puerto Rico, Mayagüez Campus, sponsored by the Howard Hughes 2000 to 2004 Undergraduate Science Education Program and the MARC and Sloan Programs, has been offering for the past four years research workshops for freshman and sophomore students. This initiative, which has had excellent outcomes, offers freshman and sophomore students from the Biology, Biotechnology and Chemistry Departments a three day cutting edge research workshop in one of the following fields: electron microscopy, molecular biology, computational biology and biochemistry. Mentoring by upper successful undergraduate MARC or Sloan students as well as discussion of undergraduate and graduate research programs and opportunities is included throughout the workshops. Participants have assessed all workshops as excellent. They consider the experience as meaningful for their academic and professional development. These workshops have motivated students into pursuing other undergraduate research opportunities, as well as make them aware of graduate studies leading to careers in biomedical science research.

1654

### Requiring Undergraduate Research for a BS Degree in Biology: Does It Do Any Good?

H. G. Kuruvilla, A. E. Schaffner, B. E. Phipps; Science and Mathematics, Cedarville University, Cedarville, OH

Undergraduate research is a wonderful tool for helping students learn the scientific method. Research provides opportunities for students to invest of their time and skills, and become part of the scientific process. Students learn valuable critical thinking skills, as well as the value of perseverance. Many graduate and professional schools look for research experience as part of their requirements for entrance into their programs. However, left to their own devices, many students will not choose to do research as part of their undergraduate career. Several years ago, the biology faculty at Cedarville University decided to make research mandatory for the BS degree in biology, leaving it as an elective for the BA

degree. Since then, we have had several classes of majors graduate. Here we provide data which shows that students appreciate being "forced" into research, and find the experience to be an important stepping stone into further education. In addition, students who have been co-authors on publications share some of their experiences related to the publishing process.

1655

**Directional Bias of Yeast Actin in Non-directional Cloning Procedure Provides a Problem-Based Approach for the Molecular Biology Laboratory**

A. Tuan, E. Jonasson, M. W. Black; Biological Sciences, California Polytechnic State University, San Luis Obispo, CA

The utility of molecular tools in a variety of disciplines has led to the design of undergraduate laboratory courses that teach the fundamentals of lab techniques and their utility in a variety of disciplines. The Molecular Biology Laboratory course taught at Cal Poly ties a variety of separate lab procedures to a common goal: the cloning and analysis of the cDNA corresponding to the yeast actin gene (ScACT1). Although many of these techniques are standard for this type of course, the use of ScACT1 has provided us with an interesting twist that promotes a problem-based approach to provide students with a self-guided, capstone lab experience. Problem: upon transforming cells with plasmids generated from a non-directional cloning procedure, all of the white colonies examined (those expected to be recombinant) were found to carry only one of the two possible orientations of the amplified ScACT1 ($<1x10^{-9}$ probability). Response: the class is led to identify 4 possible hypotheses: (1) a bias in ligation by the “non-directional” cloning system; (2) DNA sequence in ScACT1 interferes with plasmid replication in one orientation; (3) a toxic product is produced by *E.coli* from insert in one orientation; (4) students serve as the selective agent due to the analysis of only those colonies that are white, while the other orientation is propagated in blue colonies. Student groups then design relatively simple experiments to support or reject their particular hypothesis, perform these experiments, and then report the results and conclusions to the class. Contrary to student expectations, the significant directional bias demonstrated in this cloning step was due to a combination of plasmid replication interference (prokaryotic promoter elements in ScACT) and student selection (one orientation resulted in light blue colonies).

1656

**An Interactive General Biology Laboratory Manual for Latin America Students**

J. Velez-Gavilan, V. Navas, N. Diffoot, D. Delgado; Biology Dept., University of Puerto Rico-Mayaguez Campus, Mayaguez, Puerto Rico

Personnel from the Biology Department, University of Puerto Rico, Mayagüez Campus, sponsored by Howard Hughes, has written an interactive laboratory manual in Spanish to be used by freshman students in the first part of the General Biology course. The manual consists of a CD with twelve laboratory exercises that include ethics in science, the scientific method, scientific article analyses, macromolecules, the cell and those processes that occur within the cell such as membrane transport, cellular respiration, photosynthesis and molecular activities. Exercises were modified and designed to develop in students important scientific skills which include critical thinking, investigation, team work, computer and communication skills. They also aim to expose students to modern scientific issues and technology as well as emphasize ethical values. Integration of chemistry and math to biology is also emphasized. The effectiveness of the exercises included in the manual has been assessed for the past four years through the Howard Hughes curriculum enhancement program. The interactive component of the manual allows for a text with easy and direct access to photographs, to the web, to further explanations and to complementary lectures. Because of its interactive nature, procedures are illustrated by means of digital color pictures and/ or through demonstration videos which allow for easier understanding. Also, possible experimental outcomes are presented for students to accurately compare and identify their own results. The main objective for developing this laboratory manual is to offer Latin-American students a manual in their language, in an affordable CD format, that facilitates learning and understanding by offering immediate access to available information. This laboratory manual is expected to be available in spring 2006.

1657

**Genomics Perspective Provided by Undergraduate Synthetic Biology Research Using Mathematics and Molecular Biology**

A. M. Campbell,[1] L. J. Heyer,[2] K. DeCelle,[1] T. Odle,[1] N. Cain,[2] A. T. Drysdale,[1] M. P. Gemberling,[1] O. G. Hernandez,[1] A. J. Ryan[1]; [1]Biology, Davidson College, Davidson, NC, [2]Mathematics, Davidson College, Davidson, NC

Genomics and Systems Biology present new challenges to biology education. Many of the methods are expensive and data collection too massive to be reproduced by undergraduates. These challenges present a formidable barrier to bringing genomics and systems biology into the undergraduate laboratory curriculum. A new area of investigation, called Synthetic Biology, blends the perspective and interdisciplinary nature of systems biology with molecular methods available to many undergraduates. We have joined the MIT-hosted 2005 intercollegiate Genetically Engineered Machine competition (iGEM; http://parts2.mit.edu/iGEM), along with 12 other campuses in the US, Canada, and Europe. Synthetic Biology offers every biology department the opportunity to use existing resources to conduct cutting-edge research within a typical biology curriculum. Mathematics and biology can be integrated beyond simple statistical data analysis. Using iGEM resources, undergraduates designed, built, analyzed, and characterized standardized biological parts and machines that performed complex functions such as decoding and displaying results in a human readable display. Funded by HHMI, NIH, and Davidson College. Thanks to MIT's iGEM competition.

1658

**Center for Undergraduate Research Opportunities: Promoting Engagement of Undergraduate Students and Faculty in Research at the University of Georgia**

P. Kleiber,[1] M. Fechheimer[2]; [1]Center for Undergraduate Research Opportunities, University of Georgia, Athens, GA, [2]Department of Cellular Biology, University of Georgia, Athens, GA

The Center for Undergraduate Research Opportunities (CURO) was established to promote and coordinate the engagement of students with faculty in the research enterprise at the University of Georgia. CURO coordinates an array of programs, events, and classes that serve students and faculty from all disciplines and academic departments. Programs initiated with external funding from FIPSE, HHMI, or NSF have achieved institutionalization and stability within CURO (http://www.uga.edu/honors/curo/ ). CURO engages students at various stages of the research process with: 1) a web-based directory of research projects and mentors; 2) discipline-specific gateway seminars; 3) undergraduate research forums to promote interdisciplinary interaction of lower and upper division undergraduate students; 4) funded research apprenticeships for minority

students for the first four semesters; 5) honors research courses (one-on-one) for academic credit, including the thesis capstone; 6) funded summer research experiences which are immersive and intensive; 7) opportunities to share research findings at an annual statewide undergraduate research symposium; 8) publication opportunities in *the Journal for Undergraduate Research Opportunities* (JURO) and *The Undergraduate Science Bulletin* (TUSB); 9) support to attend a national research meeting such as National Conference for Undergraduate Research (NCUR) or a professional society such as American Society for Cell Biology (ASCB); and 10) encouragement for publication in peer reviewed journals. Evaluation methods using a combination of quantitative and qualitative assessment tools along with program specific and institutional data demonstrate that engagement of undergraduates in research has been dramatically enhanced since the creation of the CURO not only in biological and physical sciences, but also in other scholarly disciplines at the university. We hope that these efforts will be of interest to others engaged in similar work at other institutions.

1660

**Bio 4342, Research Explorations in Genomics: An Undergraduate Investigation of the Dot Chromosomes of Drosophila**

S. C. R. Elgin,[1] E. Slawson,[1] W. Leung,[1] M. Itano,[1] E. Kellmann,[1] C. Craig,[1] C. Shaffer,[1] J. Buhler,[2] E. Mardis[3]; [1]Biology, Washington University, St. Louis, MO, [2]Computer Science & Engineering, Washington University, St. Louis, MO, [3]Genetics & GSC, Washington University, St. Louis, MO

We have developed a genomics-based undergraduate lab course through a collaboration of the Biology Dept., Computer Science & Engineering Dept., and the Genome Sequencing Center at Washington University in St. Louis. This lab course (Bio 4342, Research Explorations in Genomics; 8 hr lab/wk) brings students together in a collaborative investigation of an outstanding problem, potentially different each year. During spring '04 and'05, students sequenced and analyzed fosmid clones derived from the dot chromosome of a strain of *Drosophila virilis*. In the first half of the semester (at the GSC) students contributed to a large data set of DNA sequencing reads and used computer-based assembly, reiterative analysis and additional sequencing to generate high quality finished sequence. The second half of the semester was focused on extensive annotation and inter-species comparison with other drosophilids. While most of the same genes are found on the dot chromosomes of D. *melanogaster* and *D. virilis*, extensive rearrangement is evident. Significant differences in the pattern of interspersed repeats are observed, with the *D. melanogaster* dot showing enrichment for DNA transposons. Coupled with other evidence, the differences in the amount and type of repetitious element present suggest a basis for the earlier observation that the dot chromosome is heterochromatic in *D. melanogaster*, but euchromatic in *D. virilis*. Long-term pedagogical goals include the development of a multi-college web-based collaborative project where larger data sets can be produced and analyzed. [We thank the student participants in Bio 4342. This work is supported by a grant from HHMI to Washington University for SCRE.]

1661

**Design of an Advanced Cell Biology Course Wherein Undergraduates Propose, Conduct, and Publish Original Research**

S. K. DebBurman; Biology, Lake Forest College, Lake Forest, IL

Undergraduate educators face significant challenges in preparing diverse graduates for a scientifically sophisticated and interdisciplinary 21st century community. Science curricula that integrate training in research with undergraduate teaching have enjoyed much funding support. The goal of providing comprehensive original research experiences within a course is a particular challenge, but was achieved in an NSF-supported advanced cell biology course described here. Students conducted individualized projects integrated to the original discovery of 86 yeast genes, which enhanced the human gene alpha-synuclein's toxicity (Willingham et al. Science 302, 1769-72, 2003). Not knowing the cellular mechanism of how these genes enhanced toxicity provided impetus for student-driven discovery. To begin with, each student picked a "my favorite gene" (MFG). Students then organized and led in-depth discussions (or lectures) to familiar each other with MFG background. Next, they uncovered current gaps in knowledge by presenting journal clubs on articles that bridged MFG with alpha-synuclein. In their quest for new knowledge, students wrote grant proposals to conduct original research based on the technologies and approaches available to them at the home institution, and they spent the first six labs mastering them. They spent rest of the semester conducting, troubleshooting, repeating experiments, and interpreting data. Instead of a traditional final exam, each wrote a primary article that was 1) published in an in-house student research journal (EUKARYON; this journal discussed as a separate poster at this meeting); and 2) submitted for publication review to national undergraduate research journals; and/or 3) presented at local or national scientific conferences. Such courses, while necessarily small and self-selective, provide successful authentic research experiences that prepare undergraduates for professional scientific careers. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

1662

**EUKARYON: Design, Publication, & Governance of a Student Peer-reviewed Undergraduate Journal of Life Science Scholarship at Lake Forest College**

S. K. DebBurman, K. Brandis, T. Vaidya; Biology, Lake Forest College, Lake Forest, IL

Inquiry-based undergraduate science education is becoming an increasingly crucial component in colleges and university curricula in the United States. The opportunity for students to publish undergraduate scholarship not only increases scientific literacy and significantly, but may motivate them towards future engagement in scientific discovery. Eukaryon is a new undergraduate research journal at Lake Forest College that publishes outstanding life science scholarship conducted by its undergraduate students. The journal's goal is to celebrate the scholarship students achieved within the College's research-rich undergraduate classrooms and student-centered research labs. The inaugural issue was published in January 2005 and featured seventeen articles in diverse categories including news and views, review articles, primary articles, research proposals, book reviews, and research proposals. Seventeen students, ranging from freshmen to graduating seniors, authored these articles. The journal is governed by a 14-member undergraduate editorial board, which not only peer review articles, but also authors the journal's editorial policies, and manages the journal's web site, with the goal to sustain Eukaryon as a truly student peer-reviewed publication that maintains high scientific journalism standards. Eukaryon website: http://www.lakeforest.edu/eukaryon (Supported by NSF-CCLI)

1663

**Undergraduate Computational Science**

G. W. Shiflet,[1] A. B. Shiflet[2]; [1]Biology, Wofford College, Spartanburg, SC, [2]Computer Science, Wofford College, Spartanburg, SC

Many significant scientific research questions are interdisciplinary in nature, involving biological and/or physical sciences, mathematics, and computer science in an area called "computational science"; and much scientific investigation now involves computing as well as theory and experiment. Despite the critical need for computational scientists, few programs and textbooks appropriate for undergraduates exist. However, computers have become fast and cheap enough; networks sophisticated enough; scientific visualization mature enough; and the Internet pervasive and friendly enough so that meaningful undergraduate computational science programs are now possible. NSF CCLI Grant 0087979 helped to fund development of an undergraduate computational science program at Wofford College, creation of extensive online educational materials (http://www.wofford.edu/ecs), and promotion of such education nationally. Biology majors have been particularly interested in Wofford's Emphasis in Computational Science (ECS), which began in 2000. ECS requirements include a B.S.; C++, Data Structures, Calculus I, Data and Visualization (C++ prerequisite), and Modeling and Simulation (calculus but no programming prerequisite); a summer internship involving computation in science. Over sixty application rich, Web-accessible course modules are used in the two computational science courses. A textbook based on these materials will appear in Spring, 2006. In the program students develop system dynamics models, such as enzyme kinetics and spread of SARS; build cellular automaton simulations, such as the immune system attack of HIV; implement algorithms, such as genomic sequence comparison; and program scientific visualizations, such as a 3D interactive animation of DNA. ECS students have had internships at such institutions as NIH, national laboratories, Greenwood Genetic Center, and various REUs. After graduation, they have obtained scientific positions in organizations, graduate, and medical schools. As Woffordâ€™s experiences demonstrate, schools can develop effective undergraduate computational science programs that provide meaningful and needed educational opportunities for students.

1664

**Frontiers in Human Embryonic Stem Cells: Advanced Lab and Conceptual Training**

C. S. Navara,[1] C. J. Redinger,[1] H. Qidwai,[1] J. D. Mich-Basso,[1] C. R. Simerly,[1] D. A. McFarland,[1] E. S. Jacoby,[1] A. Ben-Yehudah,[1] D. L. Carlisle,[1] P. J. Sammak,[1] R. A. Pedersen,[2] G. P. Schatten[1]; [1]Pittsburgh Development Center, University of Pittsburgh, Pittsburgh, PA, [2]Department of Surgery, University of Cambridge, Cambridge, United Kingdom

To realize the biomedical potentials of human embryonic stem cells (hESCs) more cell biologists and other investigators need to master the special methods for sophisticated hESC investigations. As the field of stem cell biology is young and developing, the NIH recognized a need for instruction and sharing of successful techniques and established educational grants for hosting courses that address this need. Since 2003, the Pittsburgh Development Center of Magee Women’s Research Institute has offered the Frontiers in Human Embryonic Stem Cells (FrHESC) course using federally-approved stem cell lines. FrHESC is an intensive, comprehensive, two-week lecture and laboratory training course providing hands-on experience and interactive lab sessions instructed by some of the most prominent researchers and luminaries in the field of hESC derivation, maintenance, nuclear transfer, directed differentiation and ethics. Successful culture and maintenance of hESCs can be an arduous task, however, the FrHESC course enables participants to learn valuable techniques reducing the learning curve for these procedures. More specifically, laboratory instruction includes: 1. isolation, passaging and freezing of mouse embryonic fibroblasts (MEFs) and hESCs; 2. preparation of MEF feeder plates and feeder-free plates using Matrigel®; 3. identifying undifferentiated and differentiated hESC colony morphology; and subsequent manual and enzymatic passaging of hESCs; 4. immunocytochemistry (Oct-4, SSEA1, 3, 4 and TRA1-60, 1-81) for characterization of pluripotent hESCs using; confocal imaging and epifluorescent microscopy and 5. directed differentiation experiments that coax hESC down neuronal lineages. Additionally, lectures in the past have introduced topics covering the entire gamut of stem cell research including somatic cell nuclear transfer (SCNT); therapeutic cloning; hematopoietic-, placental-, male germline-, neural-, and muscle stem cells; patient specific SCNT hESCs for disease discoveries; and ethical and legal issues of oocyte donation and embryonic stem cell derivation. Supported by the NIH

1665

**Developing Faculty-Student Collaborations: An Improvisational Performance**

R. M. Holmes; Center for Computational Science, Boston University, Boston, MA

The idea that people learn through "performing" as learners offers a fresh perspective with which to understand and practice the teaching of biology at the college level. The performance approach to learning, applied most frequently to early childhood and elementary school aged children, posits that learning is co-created and improvisational. The language of theater, improvisation in particular, is helpful to understanding the collaborative and co-creative process between faculty and students. To professional performers, improvisation refers to a set of tools and methods used to create scenes and stories without a script. In addition to spontaneity, improvisation involves listening, accepting and working with contribution of fellow performers. The improvisational challenge in science education is to include current understandings of the field without negating novices during the performance of a scientific conversation. We introduce performance and improvisation, the metaphor and practice, in the context of a research experience in which undergraduate research assistants helped to develop instructional materials on computer-based modeling of the cell cycle. The research group met once a week for an hour and a half. Improvisation games were used throughout the research experience to build the group and highlight the experience of co-building conversation. The improvisational process of accepting each other’s contributions and building a scientific conversation continued as we developed our understanding of mathematical models. Students were introduced to two mathematical models of the cell cycle (Goldbeter, 1991; Tyson, 1991) and asked to develop with the faculty an understanding of the models and materials for teaching novices. Students developed skills analyzing experimental results and organizing data in graphs and tables. A preliminary chapter on cell cycle dynamics was produced through the group’s collective, improvisational performance as researchers. Materials used and produced by the research ensemble and student perspectives will be presented.

1666

**Mathematical Modeling of the Cell Cycle: A Research Project in Undergraduate Cellular, Developmental Biology**

R. M. Holmes; Center for Computational Science, Boston University, Boston, MA

The National Research Council Committee on Undergraduate Biology Education reported in Bio2010 that the introduction of computational methods such as modeling within undergraduate biology curricula might support the development of skills needed by future biologists. ”To understand system function in biology in a predictive and quantitative fashion, it is necessary to describe and model how the system function results from the properties of its constituent elements” (National Research Council, 2002). Some biology courses are beginning to incorporate

aspects of mathematical models into their curricula (Steen, 2005). We experimented with implementing a module on numerical modeling of the cell cycle within the Cell, Developmental Biology Course at Beloit College. The overall goal of the module was to introduce students to the methods of constructing numerical models and the dynamic aspects of the cell cycle. The module was one of four projects in the research-based course taken by twenty-two students, freshmen to seniors. Text based materials consisted of excerpts from the *Cell Cycle* by Murray and Hunt, "Cell Cycle" and "Introduction to Modeling" chapters from text by Dr. Holmes (in progress) and from a list of existing mathematical models of the cell cycle. Students were asked to modify an existing minimal model of the cell cycle (Goldbeter, 1991) by including additional factors (size, binding kinetics, regulation, etc). During the project, students reviewed literature, developed their own models using *Stella*, and reported their projects as scientific articles. We present the module format and content as well as student perceptions of properties of the cell cycle and modeling as ascertained through a questionnaire using a Likert-like Scale.

1667

**Enhancing Student Learning with Tablet Technology**

M. J. Grossel, D. Eastman; Biology, Connecticut College, New London, CT

Tablet computers are being used in two courses and our peer-mentoring program at Connecticut College with the following learning objectives: (1) to integrate bench research and informatics; (2) to improve student writing; (3) to enhance classroom learning; and (4) to encourage students to seek out individual assistance. The wireless tablet technology will engage students in Genetics and Molecular Biology courses and will support our peer-mentoring program in the following manner: (1) Existing classrooms and laboratories will become integrated technology and learning environments. (2) Students will combine bench research, information literacy and database searching. (3) In the classroom, instructors will be able to write directly on projected digital images and animations. (4) Space-saving tablets will replace traditional lab notebooks, paper and pens, by allowing students to take notes directly onto digital documents. 5) Tablets will provide a unique and exciting platform for students to work together as they revise scientific papers and lab reports and when they attend peer-mentoring sessions. The expected outcomes of this new curricular design include: improved information fluency, enhanced abilities and interest in writing and critiquing papers, and better understanding of the complex concepts and mechanisms that are fundamental to genetics and molecular biology. An HP tablet will be on display at the poster.

1668

**Teaching Structural Analysis across the Biology Curriculum with Digital Microscopy**

D. R. Howard; Biology Department, University of Wisconsin-La Crosse, La Crosse, WI

The goal of this project was to provide students opportunities to learn how to obtain and interpret structural data. To accomplish this goal, digital microscopy was incorporated into a series of guided-inquiry experiments in multiple Biology courses. The project is designed to gradually develop the students' structural data skills. Over 1,300 students a year get their first exposure to digital microscopy in the Introductory Biology labs. To provide each group of 4 students a digital microscopy station at their bench, 14 student-grade trinocular compound microscopes were equipped with consumer-grade digital cameras. In addition, two digital stereomicroscopy stations are shared. In these introductory labs, students capture and label digital images in order to construct their own taxonomic keys and to analyze and present data from osmosis experiments and from genetics experiments. In the upper level Cell Biology course, 180 students use similar student-grade imaging stations to conduct histochemical investigations into the distribution of alkaline phosphatase in earthworms. In addition, Cell Biology students use two research-grade fluorescence microscopes equipped with digital cameras to analyze and present the results of immunofluorescence experiments. In the elective Microscopy course, students perform independent research projects with this equipment. In the upper level courses, students engage in more in depth image analysis and processing. Results from student and faculty surveys and external review indicate that the program is successful. Over 85% of introductory students report their imaging skills and ability to interpret structural data both improve. Over 90% of Cell Biology students indicate that digital microscopy improves both their ability to analyze data and their understanding of their results. Overall, digital microscopy allowed numerous inquiry exercises to reach their full potential for student learning. The program was supported by an NSF CCLI grant (# DUE-0127622).

1669

**An Undergraduate Science Education and Research Program in Computational Molecular Biology and Bioinformatics**

C. A. Hutton, R. P. Donaldson; Biology, George Washington University, Washington, DC

The Howard Hughes Medical Institute (HHMI) Undergraduate Science Education Program at George Washington University aims to promote undergraduate research and scholarship in Computational Molecular Biology and Bioinformatics (CMBB). Faculty and curricular development, facilities and equipment, training workshops and research opportunities facilitate interdisciplinary learning. The program's goal is to expose students to the rapidly expanding fields of CMBB study and to engage them in professional biomedical research. The program has done this by developing new courses (e.g. Bioinformatics; Biology of Proteins for freshmen) and interdisciplinary teaching modules for preexisting courses, engaging faculty in cross-disciplinary teaching, establishing scientific communication between departments, and training undergraduates in research through a summer workshop and laboratory experience. The program has altered the dynamics of scientific research at GWU: students trained in Biology work for Medical School Biochemists, Computer Scientists work for Biologists, members of the Physics and Biological Sciences departments work jointly. People from Statistics, Genetics, Molecular Biology, Microbiology and Tropical Medicine, Pharmacology, and Chemistry are also involved in the program. Importantly, it has created a cohesive community of undergraduate researchers. Crucial components of the student research program are a three week training workshop in the summer, eight weeks of research, three presentations, and opportunities to continue projects during the academic year. Students have worked in labs across the Washington, DC area: at GWU, GWU Medical School, Children's National Medical Center, National Institutes of Health, and The Institute for Genomic Research (TIGR). Of the 57 students who have participated in the summer workshop and laboratory experience, most have continued in the sciences, actively participating in research or moving on to medical school, public health, or graduate school.

1670

**Impact of Interactive Engagement on Student Learning in a Large, Advanced, Majors Biology Class**

J. K. Knight, W. B. Wood; MCD Biology, Univ. of Colorado, Boulder, Boulder, CO

We have carried out an experiment to determine whether student learning gains in a large, traditionally taught, upper-division lecture course in developmental biology, required for MCDB majors, could be increased by changing to a more interactive classroom format. In two successive semesters, we presented the same course syllabus with the same instructors using different teaching styles. In fall 2003, we used the traditional lecture format. In spring 2004, we lectured less and added classroom activities requiring student participation and cooperative problem solving, including frequent in-class assessment of understanding. We used performance on the same pre- and post-tests and on similar homework problems to estimate and compare student learning gains between the two semesters. Our results indicated significantly higher (~30%) learning gains and better conceptual understanding among students in the interactive course. To assess reproducibility of these effects, we repeated the interactive course in spring 2005 and obtained similar results. B students registered the largest increases in learning gains between the two types of classes. Student attitudes toward the interactive style of instruction were mixed, probably partly because the students were primarily seniors whose previous large classes in the major were taught as traditional lecture courses. Our findings parallel results of similar teaching-style comparisons made in other disciplines. On the basis of this evidence, we propose a general model for teaching of large biology courses that incorporates interactive engagement and cooperative work in place of some lecturing, while retaining course content by demanding greater student responsibility for learning outside of class.

1671

**Prototype Textbook for Teaching Cancer Biology to Non-science Majors**

P. K. Hanson; Biology, Birmingham-Southern College, Birmingham, AL

Education research has documented several factors that hinder learning in introductory science courses, including excessive use of jargon, failure to relate to other disciplines, emphasis on content rather than process, lack of a unifying concept, and irrelevance to life outside the classroom [1-3]. Thematic courses help overcome many of these problems by providing a central topic (cancer, AIDS, the physics of music, etc.) that is interdisciplinary and relevant to students' development into educated, proactive citizens, one of the central goals of a liberal education [4]. Despite these advantages, the resources available for thematic courses are scattered at best, and at worst they are out of date or incorrect. To overcome this problem for non-majors courses in cancer biology, I have developed a prototype textbook that uses an interdisciplinary, evidence-based approach to acculturate students to the process of science while teaching them about the origins, diagnosis, treatment, and prevention of cancer. This interdisciplinary text explores statistics (what does cancer "risk" really mean?), physics (the nature of the electromagnetic spectrum and its role in causing, detecting, and treating cancer), as well as the ethical, legal, and social implications of the disease. Preliminary student feedback indicates the textbook helps students understand the process of science by encouraging them to critically evaluate evidence and arrive at educated conclusions. 1. (1996) *Shaping the Future*, Washington, D.C.: National Science Foundation. 2. Sundberg, M.D. et al. (1994). Decreasing Course Content Improves Student Comprehension of Science and Attitudes toward Science in Freshman Biology. J. Res. Sci Teach. *31*: 679-693. 3. Stover, S. and M. Mabry. (2005). Merging Science and Society: An Issues-Based Approach to Nonmajors Biology. J. Coll. Sci. Teach. *34*: 40-43. 4. (2002) *Greater Expectations*. Washington, D.C.: Association of American Colleges and Universities.

1672

**The Nature of Life: An Orientation Course That Introduces Freshmen to the Disciplines of Biology, Builds Community, and Teaches Strategies for Success in College**

R. Wright, J. S. Anderson, D. Biesboer, S. Cotner, S. Gilbert; College of Biological Sciences, University of Minnesota, Minneapolis, MN

The University of Minnesota aims to increase the retention and graduation rates of entering freshmen. To help accomplish this goal, the College of Biological Sciences has developed a one-credit orientation course called "The Nature of Life" that is required for all of its incoming freshmen, a class of ~350 students. The Nature of Life program introduces students to the breadth of biological disciplines, acquaints them with college and university resources, and helps them become integrated into a community of learners. The program begins with a four-day experience at the Itasca Biological Station and Laboratories located at the headwaters of the Mississippi River. During this time, students participate in three different interactive learning modules that focus on topics ranging from bioethics and neurobiology to aquatic ecosystems and spatial modeling. Additional programming includes plenary lectures and discussions about career planning, time management, research involvement, campus resources, and University of Minnesota traditions. A college-level exam is given, graded, and returned to students on the last evening of the program, followed by a discussion of strategies for more effective learning. To receive credit for the course, students must also prepare three reflective essays, attend events on campus, and participate in discussions of articles from a popular science magazine. First semester freshmen who participated in the Nature of Life program have higher overall GPAs and fewer failing grades than those of previous years. In addition, one year retention in the college increased from 66% to 81%. To our knowledge, this program is unique among research universities in the United States and provides a model for supporting a successful transition of students from high school into the academic challenges of the university.

1673

**Teaching the Cognitive Elements in Scientific Reasoning to Students of Cell Biology**

W. S. Bradshaw,[1] S. Reeve,[1] J. Bell,[2] R. Sudweeks[3]; [1]Microbiology and Molecular Biology, Brigham Young University, Provo, UT, [2]Physiology and Developmental Biology, Brigham Young University, Provo, UT, [3]Educational Psychology and Technology, Brigham Young University, Provo, UT

The objectives of a course in cell biology course can well include an explicit effort to promote the acquisition of scientific thinking skills. Our specific focus is the development of the ability to draw conclusions from experimental data, particularly those that support the most fundamental conceptual principles in the field. This skill is assessed by the ability of students to write sentences that demonstrate their ability to interpret figures and tables and answer the questions that investigators have posed through the use of specific experimental protocols. We believe this ability is not intuitive for most people, and is rarely required in high school or undergraduate courses. We report here experimental attempts in the classroom to identity those separate components of analytical thinking that are practiced in cell biology. The existence of these variables has been demonstrated through the use of statistical methods such as generalizability analysis and the occurrence of unique patterns in exam score distributions. Problems have been constructed that distinguish the need for background conceptual knowledge from those that don't because the data are self evident for the person possessing "figure literacy." Other problems reveal some of the conditions under which the student is able to exhibit transfer, the ability

to apply prior understanding of principles in an unfamiliar setting. The results of surveys of students' affective response to this kind of problem solving also suggest separable attributes of the thinking process. We conclude by reporting attempts to design pedagogical strategies that promote improvement in these various aspects of scientific reasoning.

1674

**Rethinking Exams and Letter Grades: How Much Can Teachers Delegate to Students?**

W. S. Bradshaw,[1] J. Bell[2]; [1]Microbiology and Molecular Biology, Brigham Young University, Provo, UT, [2]Physiology and Developmental Biology, Brigham Young University, Provo, UT

Modification in the design of a cell biology course have been made in order to promote metaconitive assessment and provide formative feedback. Traditional midterm exams were replaced by weekly formative assessment problems, solved under exam-like conditions, which were accompanied by extensive self-, peer-, and instructor-review. The routes to final letter grades were defined by demonstrated competence with tasks at various levesl of analytical complexity. These include comprehension of conceptual models and biological mechanisms, and solving problems requiring the interpretation of experimental data. A mechanism was provided by which students could evealuated their cumulative performance , and then propose and negotiate a letter grade. We report the positive impact of this design on measures of both student performance and affect.

1675

**An Inquiry-, Experience(and Exercise)-, and Observation-Based Understanding of Integration of Cell and Human Biological Systems for Integration of Cell Biology and Health Education**

Y. Atomi, S. Matsuo, T. Sakurai; Dept Life Scis, Univ Tokyo, Tokyo, Japan

Paradoxically a progress of cell and molecular biology is promotion to increasing of life-style-relating diseases in recent years. Understanding of own cell (life) system working his/her body may contribute to cell biology education as well as health education. For undergraduate students we have supplied Sport Science Course before five years ago. In this course, students learns self system through dynamicity at different striatum of self-individuals, functional tissues in mammalian, and cells in mammalian tissues and cultured cells on dishes. Introductions of self-recognition, exercise physiology for self body in early stages of the course are effective for extracting motivation of learning and understanding self biological system of the body. Successful orders of understanding are 1) Integrative brain system and disagreement of his/her intension and the output, 2) Supporting system of endurance running from hear rate (linear) and respiratory (non-linear phenomena) responses to gravity (posture) and dynamic/static exercises, 3) Observations of mammalian tissues from cellular basis using fluorescent dyes, and 4) Beating cultured heart cells and muscle differentiation process from myoblasts to myotube, and neuronal cells, which constituting a use-dependent brain integrating system of human body and mind educating facing oneself. Especially beating heart cells and a process of muscle differentiation are appropriate for understanding autonomous, self-propagating chemical system constituted by fibrous protein cytoskeleton and motor system developing tension from cell to human body. A principle of a use dependency in brain and muscle function at cellular level is educable hypothesis for self-oriented health education.

1676

***The God Gene* in the Classroom: An Analysis of VMAT2 Polymorphisms in a Non-majors Undergraduate Laboratory Class**

L. A. Silveira; Biology Department, University of Redlands, Redlands, CA

In his book *The God Gene* (Doubleday, 2004), Dean Hamer describes an A/C polymorphism in the human VMAT2 gene that correlates weakly with self-transcendence scores on a psychological test. VMAT2 encodes a monoamine transporter that packages neurotransmitters into vesicles in the brain. Hamer proposes that variations in this gene and others render one more or less open to spiritual experiences. In an interdisciplinary undergraduate course on biotechnology we used *The God Gene* as a springboard for discussing concepts in genetics and biotechnology. In the laboratory, we replicated Hamer's study on a smaller scale. The students, mostly sophomores majoring outside the sciences, isolated DNA from their cheek cells, used PCR to amplify a region of VMAT2, purified the amplified DNA, sent it to a facility for sequencing, and analyzed the sequence at the sites of three polymorphisms. We compiled the data, then the students conducted a statistical test to see if any of the polymorphisms correlated significantly with self-transcendence or other aspects of personality measured by the psychological test. In the classroom, we discussed the Central Dogma, protein function, mutation, basic Mendelian genetics, simple and complex traits, neuron function, experimental design, and peer review, as well as each of the laboratory techniques. Pre- and post-tests assessed student learning and attitudes. Students embraced the project enthusiastically and gained in understanding of the core concepts. By the end of the project students were able to choose a complex trait and, in a final paper, make a case for or against a genetic contribution to that trait.

## Actin-Associated Proteins III (1677-1697)

1677

**The Tobacco LIM Protein NtWLIM1 Stabilises the Actin Cytoskeleton against Depolymerisation by Latrunculin B**

C. Hoffmann, C. Thomas, A. A. Steinmetz; Plant Molecular Biology, CRP-Sante, Luxembourg, Luxembourg

NtWLIM1 is one of six LIM domain proteins identified in tobacco. Transcripts of the gene have been detected in all organs of the tobacco plant, but not in pollen grains and cell suspension cultures. Initial attempts to express the protein in fusion with GFP and under the control of the cauliflower mosaic virus 35S promoter failed to generate transgenic cell lines as well as tobacco plants, suggesting that an overproduction of the protein interferes with an essential cellular function. Therefore, in order to study the subcellular localisation of the protein, we have placed the fusion construct under the control of an inducible promoter in the plant transformation vector pTA7002 (Aoyama and Chua, 1997). The recombinant plasmid was then introduced into tobacco BY2 cells using Agrobacterium-mediated transformation, and recombinant cell lines were selected on appropriate medium. We observed that NtWLIM1 exhibits a dual nuclear and cytoplasmic distribution. In the cytoplasm it was predominantly associated with a filamentous network identified as the actin cytoskeleton by co-labeling experiments as well as by treatment of the cells with latrunculin B, a drug that specifically disrupts actin filament assembly. Importantly, we noticed that the ratio of actin-associated NtWLIM1 relative to the unbound protein varied with the culture conditions: for instance, a reduction of cell oxygenation significantly enhanced

the binding of NtWLIM1 to microfilaments. In addition, when these cells were treated with latrunculin B and subsequently analysed at different times after addition of the drug, the actin cytoskeleton was still clearly visible after 40 minutes while in control cells not expressing the protein the filamentous structures had completely disappeared. These data indicate that binding of the NtWLIM1 protein to the actin cytoskeleton stabilizes the latter against depolymerisation by latrunculin B.

1678

**Characterization of Role of LIM and SH3 Protein-1 (Lasp-1) in Epidermal Growth Factor (EGF) Endocytosis and Macropinocytosis**

T. E. Rajapaksa,[1] C. S. Chew,[2] C. T. Okamoto[1]; [1]Department of Pharmaceutical Sciences, University of Southern California, Los Angeles, CA, [2]Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA

Lasp-1 is a ~40 kDa multidomain protein that is overexpressed in several breast cancer cell lines and normally expressed at relatively high concentrations in a select number of secretory epithelial cells and in certain brain cells. Lasp-1 co-localizes with F-actin and the endocytosis-regulating protein, dynamin, in several cell types, particularly in regions of active remodeling of the plasma membrane, such as filopodia and lamellipodia. In this study, we sought to determine if lasp-1 plays a role in regulating receptor-mediated endocytosis by assaying EGF uptake in HeLa cells transfected with wild type lasp-1 or with wild-type lasp-1 fused to enhanced Green Fluorescence Protein tagged (Lasp-eGFP). Under both transfection conditions, there was an increase in punctate EGF-containing structures compared to those present in untrasfected and mock transfected cells. These structures were surrounded by a "coat" of Lasp-1 staining. In addition, lasp-1 transfection was correlated with the appearance of large (~3-5µm diameter) EGF-containing structures that were morphologically similar to macropinosomes. This observation led us to investigation of possible role of lasp-1 in regulating macropinocytosis. TRITC-dextran uptake was analyzed with or without EGF stimulation in untransfected and mock transfected HeLa cells as well as in cells transfected with different lasp-1 constructs. Again, cells transfected with wild-type lasp-1 showed increased formation of macropinosomes compared to untransfected and mock transfected cells. These results support a role for lasp-1 in the regulation of macropinocytosis in HeLa cells.

1679

**Chromosomal Assignment of Lasp1 and Lasp2 Genes and Organization of the Lasp2 Gene in Chicken**

A. G. Terasaki,[1] H. Suzuki,[1] J. Ando,[2] Y. Matsuda,[2] K. Ohashi[1]; [1]Chiba University, Chiba, Japan, [2]Hokkaido University, Sapporo, Japan

Lasp-1 and lasp-2 are actin-binding proteins that contain a LIM domain, two nebulin repeats and an SH3 domain with significant identity. We determined the chromosomal locations of the LASP1 and LASP2 genes in chicken by fluorescence in situ hybridization. The LASP1 gene was localized to a pair of microchromosomes and the LASP2 gene was localized to chromosome 2p3.1, indicating that the chromosomal locations of the LASP1 and LASP2 genes are highly conserved between chicken and human. The comparison of genomic and cDNA sequences of chicken lasp-2 and nebulette, a nebulin-related protein in muscle, suggested that both the corresponding mRNAs shared exons in the same manner as their human homologues. When compared with the domain structure of nebulette, another nebulin repeat was predicted for lasp-2, and all the nebulin repeats of lasp-2 were better conserved than those in nebulette. We also found the exon boundaries in nebulin repeats of lasp-2 were similar to those of other nebulin-related proteins.

1680

**LIM Domain Proteins from Arabidopsis thaliana**

C. Thomas, M. Dieterle, C. Hoffmann, A. Steinmetz; Plant Molecular Biology, CRP-Sante, Luxembourg, Luxembourg

The Arabidopsis genome encodes six CRP-related LIM domain proteins three of which are specifically and abundantly expressed in mature pollen (AtPLIM1, AtPLIM2 and AtPLIM3), while the other three (AtWLIM1, AtWLIM2 and AtWLIM3) are expressed in the vegetative tissues. We are investigating the function of AtWLIM1 (At1g10200) in various tissues of the Arabidopsis plant. Microarray data have shown that of the three WLIM genes this is the most abundantly expressed in Arabidopsis (Zimmermann et al., Plant Physiol. 136:2621-2632, 2004; https://www.genevestigator.ethz.ch/). We have analysed the subcellular localisation of the protein following inducible expression of the GFP-fusion protein in transgenic Arabidopsis seedlings. Laser scanning confocal microscopy observations showed that the fusion protein associates with filamentous structures in several cell types. Following incubation of the seedlings with Latrunculin B the cortical cytoskeletal structures disappeared while the more internal structures appeared to be more resistant. This sensibility of the LIM-GFP-labeled cables to Lat B identified these structures as actin filaments. Interestingly also, while in root hairs the cytoskeletal organisation was identical to that observed for fimbrin-GFP-expressing cells, in another root cell type marked differences in cytoskeletal structure were observed : most notably, the AtWLIM1-GFP expressing cells showed thick, more randomly organized actin cables, whereas in fimbrin-GFP-expressing cells the cables were much thinner and longitudinally oriented. These data suggest that plant LIM proteins represent new actin cytoskeleton organisers that can stabilize the actin cytoskeleton network.

1681

**Coronin Functionally Interacts With Cofilin And Arp2/3 Complex To Regulate Actin Dynamics**

M. Gandhi, H. I. Balcer, B. Goode; Rosentiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA

Coronins are a conserved family of actin-associated proteins that contain an N-terminal WD-repeat region and a C-terminal coiled-coil (CC) domain. The WD-repeat region of yeast coronin (Crn1) mediates high affinity binding interactions with F-actin and a modest ability to accelerate actin filament assembly in vitro by an unknown mechanism (Goode et al., 1999). In contrast, the CC domain of Crn1 is required for homo-oligomerization, bundling of actin filaments, and direct spatial inhibition of Arp2/3 complex (thought to promote filament branching) (Humphries et al., 2002). Here, we report a novel functional interaction that provides mechanistic insights into how coronin regulates actin dynamics in cells. Through its WD-repeat-containing region, coronin synergizes with cofilin biochemically and genetically to sever actin filaments and accelerate actin turnover in vivo. The new activity we describe for coronin synergizing with cofilin explains the ability of Crn1 to promote actin assembly in vitro. Further, it is consistent with genetic interactions between crn1 and cof1-22 and act1-159. Using a charged-to-alanine mutagenesis approach, we are now mapping the actin-binding site(s) on the WD-repeat region of coronin. We have also dissected the CC domain and identified a single residue that when mutated suppresses the lethality caused by CRN1 overexpression. Thus, coronin regulates actin dynamics by two distinct yet

possibly integrated activities, functional synergy with cofilin in promoting filament turnover and spatial regulation of Arp2/3 complex activity.

1682

**Effect of Yeast Arp2/3 Complex on Muscle/Yeast Hybrid Actins**

P. A. Rubenstein, K. Wen, M. McKane; Biochemistry, University of Iowa College of Medicine, Iowa City, IA

Arp2/3, in combination with an activating protein such as N-WASP, creates filament branches leading to enhanced rates of actin polymerization. We recently demonstrated that yeast Arp2/3 (yArp2/3) alone efficiently facilitates polymerization and branching of yeast actin, although with muscle actin, an activating factor is required. Conversely, bovine Arp2/3 requires an activating factor to affect both yeast and muscle actins. To investigate the molecular basis for the difference in activity of yArp2/3 toward yeast and muscle actins, we constructed yeast strains expressing hybrid muscle/yeast actins as their only actins. One hybrid (sub1) has all 18 of the muscle specific residues in subdomain 1 except that the muscle 4-acidic residue N-terminal fragment contains only 3 acidic residues. The second hybrid, (sub1,2) contains in addition the 3 subdomain 2 muscle specific substitutions. Both strains exhibit a mildy decreased maximum cell density in complete medium and die at 37$^{o}$. Both strains grow on glycerol as sole carbon source indicating normal mitochondrial function. By phalloidin staining, both strains appear to have lost actin filaments, and patches are depolarized. Abnormal actin assemblies are seen in both strains but appear more prevalent in the sub1,2 cells. In the presence of excess ATP, both purified hybrid actins polymerize relatively like WT actin. In the absence of excess ATP, unlike WT actin, both actins begin to polymerize and then depolymerize suggesting a greatly increased critical concentration for ADP-actin. yArp2/3 alone will not facilitate polymerization of sub1 actin but will in the presence of N-WASP VCA. With sub1,2 actin, neither yArp2/3 alone nor with the VCA fragment will facilitate actin polymerization. These results show that differences in subdomain 1 and 2 surface residues alone are insufficient to explain the difference between the action of yArp2/3 on yeast vs. muscle actins.

1683

**Structure of the Arp2/3 Complex Branch on F-actin**

D. Sept, A. R. Rammohan; Biomedical Engineering, Washington University, St. Louis, MO

The arp2/3 complex is a central player in the dendritic nucleation model since it branches off of exisitng actin filaments and nucleates the growth of new filaments There has been little information about the molecular level interactions between the arp2/3 complex and F-actin but the key proteins in the arp2/3 complex have been identified as p20 and p34. Through detailed computational studies using a combination of molecular dynamics and molecular docking simulations, we have developed a model for the arp2/3 complex branch structure. Indeed we find the prime contacts between the complex and F-actin are via p20 and p34 with p16 making additional interactions. Our model is in excellent agreement with parallel mutagenesis studies and provides some hints about the activation pathway.

1684

**Unravelling the Multiple Levels of SCAR/WAVE Regulation**

A. Y. Pollitt, R. H. Insall; School of Biosciences, University of Birmingham, Birmingham, United Kingdom

Cells move by protruding and retracting pseudopods, which are principally made of branched actin filaments. These networks are formed by the activity of the Arp2/3 complex. SCAR/WAVE proteins are important activators of the Arp2/3 complex. SCAR/WAVE has recently been shown to form a macromolecular complex with four other proteins: PIR121, Nap1, Abi2 and HSPC300. There is still much controversy about the mechanism of SCAR/WAVE regulation. We have therefore generated gene knockouts of individual SCAR complex members in *Dictyostelium discoideum* to investigate their roles in the function and regulation of SCAR *in vivo.* Unexpectedly they all produce distinct phenotypes: • PIR121 null cells display a complex, dominant active SCAR phenotype • Nap1 null cells are more severely affected than SCAR null cells and possess an adhesion defect not seen in other mutants. • HSPC300 null cells are comparable to SCAR null cells. • Abi null cells exhibit a cytokinesis defect not seen in other mutants. This implies that the pathways that regulate SCAR/WAVE are more complex than previously thought. Additionally some complex members, in particular Nap1, form part of additional pathways independent of SCAR/WAVE. We are investigating the pathways behind each complex member and their relationships to SCAR/WAVE regulation. We have also demonstrated that SCAR/WAVE is phosphorylated in multiple positions providing a further important level of regulation.

1685

**Loss of Scar1 in Mouse Embryonic Fibroblasts Does Not Detectably Affect the Actin Cytoskeleton**

J. A. Legg; Biosciences, Birmingham University, Birmingham, United Kingdom

Scar1, a member of the Wiskott-Aldrich syndrome protein family, has been identified as an activator of the Arp2/3 complex, leading to the formation of new actin filaments. The aim of this study was to determine the importance of Scar1 in the formation of actin structures, such as filopodia, lamellipodia and dorsal ruffles. Here, we report a comparative analysis of actin structure formation when comparing wild-type and Scar1 knock out primary mouse embryonic fibroblasts. In cell spreading assays on fibronectin and laminin substrates, both the wild-type and Scar1 null cells spread at the same rates and produced similar actin protrusions. The motility of the cells was also comparable, as determined by using wound-healing assays. Using platelet derived growth factor to stimulate the formation of circular dorsal ruffles, both the wild-type and Scar1 knock out primary cells peaked in dorsal ruffle formation after 5 minutes and the same percentage of cells responded to growth factor stimulation at each time point. The ability of fibroblasts to produce multiple dorsal ruffles per cell was also not impaired by the loss of Scar1. When these primary cells were compared to immortalized wild-type and Scar1 null mouse embryonic fibroblasts cell lines, although the immortalized cells displayed a hypersensitivity to platelet derived growth factor stimulation, neither set of cells displayed a phenotype in dorsal ruffle formation. Taken together, these data suggest that there is a redundancy amongst the Scar protein family and that Scar1 is not essential for the formation of dorsal ruffles or lamellipodia in fibroblasts.

1686

**Calcineurin Dephosphorylates the C-Terminal Region of Filamin in an Important Regulatory Site: A Possible Mechanism for Filamin Mobilization and Cell Signalling**

E. Garcia, D. J. Jay; Cardiovascular Biomedicine, National Institute of Cardiology, Tlalpan, Mexico

Calcineurin is a calcium/calmodulin-dependent threonine/serine protein phosphatase which is required for the dephosphorylation and subsequent translocation of nuclear factor of activated T cells (NFAT) proteins from the cytoplasm to the nucleus in most immune system cells. Inactivation of calcineurin by cyclosporin A, however, can also alter a spectrum of other cellular functions in cells outside the immune system. The results of the present study indicate that a purified C-terminal recombinant region of the cytoskeletal protein filamin A is a suitable substrate for calcineurin in vitro. Furthermore, 1 μ M cyclosporin A reduced the dephosphorylation of the C-terminal recombinant fragment in 293FT cells. Site directed mutagenesis analysis showed that the dephosphorylation steps occurred in serine 2152 which was previously shown to provide resistance to calpain cleavage when endogenous PKA is activated. In contrast, phosphorylation of Ser2152 was recently reported to be necessary for membrane dynamic changes. In this regard, we report that cyclosporin A protects in a doses-dependent manner native filamin in platelets from calpain degradation. The results presented here could be combined with the available information in a single model, assuming that some of the peptide fragments released by calcineurin-regulated calpain action could participate as "signaling integrator proteolytic peptides" (SIPP) mediating actions in downstream pathways, with implications to other cellular routs like insulin signaling, androgen receptor translocation or immune response, which may help to explain the controversies reported on the role of filamin phosphorylation in actin dynamics. The possibility that other cytoskeleton-associated proteins like tau could function in a similar fashion is discussed.

1687

**Evolution of Specificity in Cytoskeletal Remodeling by Diverging the Activation Domains of MIM and ABBA**

J. Jin, R. Gonzalez-Quevedo, B. Strong, J. Hui, A. E. Oro; Program in Epithelial Biology, Stanford University, Stanford, CA

Remodeling of the actin cytoskeleton drives the migration, invasion and proliferation of epithelia. A consortium of modular actin-binding, polymerization, and bundling proteins coordinate development and tumor invasion, but how specific spatial rearrangments occur in response to global and local patterning signals remains poorly understood. We have previously identified the inducible actin bundling protein Missing in Metastasis (MIM), whose levels are controlled by the morphogen Sonic hedgehog. MIM activity at the membrane is controlled by its activation domain, sequences distinct from the actin bundling domain, binding to the cytoplasmic tail of Receptor Protein Tyrosine Pshosphatase (RPTP) delta. Here we show how these modular regulatory proteins evolve by examining the function of ABBA, a closely related protein that shares with MIM 95% identity in the IMD actin bundling domain and 55% identity in the activation domain. Both proteins alter the actin cytoskeleton by cross-linking individual actin filaments to form thick bundlings thus promoting cell adhesion. The IMD domains appear interchangeable, as they readily form heterodimers. In contrast to MIM, levels of ABBA RNA are not responsive to Shh signaling, but instead appear to be more uniformly distributed in the tissues. Moreover, MIM and ABBA have distinct activation domains that respond to different membrane signals. MIM binds and relocalizes RPTPdelta to the plasma membrane while ABBA responds to a non-RPTPdelta protein. Chimeric protein analysis demonstrates that the MIM activation domain confers the specificity to interact with and relocate RPTP delta. Our data show how small alterations in the activation domain can confer new cytoskeletal re modeling paradigms during evolution.

1688

**Identification of a cAMP-Dependent Protein Kinase Phosphorylation Site in the N-Terminal Region of Human Filamin**

D. J. Jay, R. Uribe, E. Garcia; Cardiovascular Biomedicine, National Institute of Cardiology, Tlalpan, Mexico

Filamin A (FLNa) cross-links actin filaments (F-actin) into three-dimensional gels in cells, attaches F-actin to membrane proteins, and serves as an integrator of cell mechanics and signalling by collecting diverse proteins. Previous studies indicated that a dynamic phosphorylation/dephosphorylation process may modulate the interaction of ABP with other cytoskeletal elements. In this regard, the analysis of the amino acid sequence of ABP revealed multiple consensus sequences specific for diverse kinases including three sites for cAMP-dependent protein kinase (PKA) at residues 167, 2152 and 2336. In this study an N-terminal region of the cytoskeletal protein FLNa was subcloned and expressed in the pT7-7/NT - E. Coli BL21 (DE3) system. As predicted by the aminoacid sequence, this recombinant fragment, a 26 kDa peptide containing the actin-binding domain, could be phosphorylated by the catalytic subunit of PKA. Site-directed mutagenesis analysis confirmed that $^{32}P$ incorporation occurred in threonine 167, the only potential PKA phosphorylation site predicted to be present in this region of the molecule. This residue belongs to a threonine-proline motif and was previously suggested to be involved in regulating F-actin interactions even though its phosphate content was not apparent in native FLNa. Results also showed that phosphorylation of the ABP fragment resulted in an increased interaction with a low-speed sedimentable complex obtained from Triton X-100-solubilized platelets (platelet cytoskeleton). Possible implications of the phosphorylation of this site are discussed in terms of the role of filamin in cytoskeleton reorganization.

1689

**Image-based Searches for the Molecular Targets and Pathways Damaged by Sulfur Mustard**

R. J. Werrlein, C. R. Braue, J. F. Dillman; Cell and Molecular Biology, USAMRICD, Aberdeen Proving Ground, MD

To determine how the blistering agent sulfur mustard (SM, bis-2-chloroethyl sulfide) disrupts epidermal basal-cell attachments in human skin, we have been tracking the development of postexposure lesions by using non-invasive multiphoton microscopy, high-resolution immunofluorescent imaging, and gene-array analysis. From the onset of preclinical lesions in human epidermal keratinocytes (HEK), we have found that SM induces early and progressive disruptions of F-actin organization and of mRNA expression that are coincident in space and time. The coincident changes in gene expression include 5 of 13 myosin I isoforms, 1 of 3 myosin II isoforms, Cdc 42, $\beta_4$ integrin and other proteins that localize and concentrate in areas of dynamic HEK attachment (lamellipodia, filopodia) and of rapid actin reorganization. These characteristic changes occur within 15 minutes of exposure, i.e., early in the “clinical latent phase” of toxicity, and well in advance of vesication. With the aid of these preclinical biomarkers, we have produced image-based search strategies that may link the onset of SM toxicity to actin-coupled signaling pathways.

1690

**Non-muscle Caldesmon Plays a Role in Actin Stress Fiber Disassembly**

J. Kordowska, S. Jiang, C. Wang; Muscle and Motility Group, Boston Biomedical Research Institute, Watertown, MA

Previously we have found that depending on the state of the cell, non-muscle caldesmon (l-CaD) assumes different distributions: In quiescent, well-spread cells unphosphorylated l-CaD is closely associated with organized actin cytoskeleton such as stress fibers and helps to maintain the cell

shape. In stimulated cells l-CaD is phosphorylated and moves to nascent focal adhesions where further modification may occur in order to enable cell attachment to the substratum (Kordowska and Wang, Mol. Biol. Cell, 15, S802, 2004). This potentially regulatory function of l-CaD was tested by expressing GFP-tagged, non-phosphorylatable mutants in cultured rat aortic fibroblast cells. Over-expression of either wild-type l-CaD or Ser-to-Ala mutants at the two ERK sites (Ser497 and Ser527) resulted in cells with thicker actin bundles and more prominent focal adhesions. Treatment with phorbol 12-myristate 13-acetate (PMA) induced disassembly of actin stress fibers and focal adhesions in cells expressing wild-type l-CaD more extensively than in untransfected cells. Contrary to this, mutant-expressing cells exhibited less disassembly of actin stress fibers and focal adhesions upon PMA stimulation compared to untransfected cells or cells transfected with pEGFP alone. Taken together, these observations suggest that exogenous, unphosphorylatable l-CaD is able to compete off endogenous l-CaD and occupies actin stress fibers, thereby preventing quick disassembly even in the presence of stimulants. Our data also suggest that over-expressed wild-type l-CaD becomes phosphorylated upon PMA stimulation; such phosphorylated protein plays an active role in promoting disassembly of actin cytoskeleton. Supported by NIH (P01 AR41637).

1691

**Filamin A Plays a Role in the Stress-Stiffening Response of Melanoma Cells**

K. E. Kasza,[1] S. Hu,[2] F. Nakamura,[3] T. P. Stossel,[3] N. Wang,[2] D. A. Weitz[1]; [1]DEAS, Harvard University, Cambridge, MA, [2]Physiology Program, Harvard School of Public Health, Boston, MA, [3]Hematology Division, Brigham and Women's Hospital, Boston, MA

Filamin A (FLNa) is an abundant protein in vertebrate cells that cross-links actin filaments and connects the cytoskeleton to the cell membrane. Disruption of FLNa expression impairs cellular motility and membrane stability. Reconstituted gels of short actin filaments cross-linked with FLNa mimic many of the mechanical properties of cells, while actin filaments cross-linked with other proteins do not. The dramatic stiffening of these gels in response to applied stress closely replicates cellular stiffening behavior. The molecular origins of cell mechanical behavior is poorly understood, and it is unclear whether filamin plays a similar dominant role in the cell as it does in reconstituted networks. To investigate this, we compare the role of FLNa in the stress-stiffening response of a human melanoma cell line (M2) deficient in FLNa with a transfected subline (A7) expressing FLNa. We probe cell stiffness using several techniques, including magnetic twisting cytometry (MTC) and atomic force microscopy. We determine the level of contractile stress in the cytoskeleton using traction force microscopy and also apply stress to cells using MTC. Cells are plated on polyacrylamide substrates of physiological stiffness, coated with extracellular matrix (ECM) proteins. On soft substrates, we find that A7 cells both exert traction forces and have stiffnesses several times larger than M2 cells do. Further, we find that as the stress applied to cells with MTC is increased, the mean stiffness of A7 cells increases by several times while the mean stiffness of M2 cells remains relatively constant. This effect is robust across a variety of different substrate stiffnesses coated with various ECM proteins. These results are consistent with data from the reconstituted network, and support the hypothesis that the filamin-actin network is a key cytoskeletal structure mediating the stress-stiffening response in cells.

1692

**Filopodia Formation in Neuronal Cells**

F. V. Korobova, T. M. Svitkina; Department of Biology, University of Pennsylvania, Philadelphia, PA

We recently proposed a convergent elongation model for filopodia formation, which suggests that filopodia are formed by reorganization of the branched actin network into bundles. It remains unclear whether this model is applicable to growth cones of neuronal cells, which are extremely rich in filopodia, but apparently do not display conventional dendritic network. Here we analyzed filopodia initiation in primary Xenopus neurons and two neuroblastoma lines. By electron microscopy, growth cones contained abundant filopodial bundles, but the veils between bundles had dendritic organization and contained Arp2/3 complex and cortactin. Kinetic analysis of filopodia initiation by phase contrast or fluorescent microscopy after YFP-actin expression (in neuroblastoma cells) revealed that filopodia originated either from veils between pre-existing filopodia in growth cones, or from small lamellipodia transiently formed on minor neurites. Filopodia formation began as condensation of phase-dense material (or YFP-actin) in the lamellipodia/veil interior followed by appearance of a bump at the cell edge, which then quickly protruded making a filopodium. Lamellipodia-to-filopodia transition was very fast and completed within a few seconds. Correlative electron microscopy of forming filopodia revealed dendritic organization of actin at sites of the initial condensation of material and at the bases of bumps and nascent filopodia. The patches of dendritic network giving birth to filopodia were very transient and could not be detected at filopodial roots a few seconds after their formation, probably, because of turnover. Thus, our findings indicate that filopodia formation in neuronal cells also occurs by the convergent elongation mechanism. However, the transformation of the dendritic network to filopodia is extremely efficient in these cells and requires a limited amount of network to form a large number of filopodia. Supported by NIH grant GM 070898.

1693

**Missing in Metastasis Interprets Global and Local Patterning Signals to the Actin Cytoskeleton**

A. E. Oro, R. Gonzalez-Quevedo; Program in Epithelial Biology, Stanford University, Stanford, CA

During development, dynamic remodeling of the actin cytoskeleton allows the precise placement and morphology of tissues. Morphogens such as Sonic hedgehog (Shh) and local cues such as receptor protein tyrosine phophatases (RPTPs) mediate this process, but how these signals result in precise spatial regulation of the cytoskeleton is poorly understood. We previously identified *Missing in Metastasis* (*MIM*) as a Shh-inducible, WH2 domain-containing protein that, together with Gli, can recapitulate Shh effects in regenerated human epidermis by potentiating Gli transcription (Genes and Dev 8:2724; 2004). We have extended our studies to show that MIM is an inducible actin-bundling protein whose levels are controlled by Shh. MIM-dependent cytoskeletal remodeling and stable cell extensions require an activation domain distinct from sequences required for bundling *in vitro*. This activation domain binds RPTPδ and directs both MIM and RPTPδ membrane localization. MIM-dependent cytoskeletal changes can be inhibited using a soluble RPTPδ-D2 domain. Enhanced F-actin deposition occurs in a MIM-dependent manner when RPTPs are crosslinked using an antibody to the extracellular domain, suggesting RPTP ligand binding provides additional levels of specificity. Our data suggests how *MIM* links global patterning morphogens like Shh and paracrine signaling pathways like RPTPs to induce precise cytoskeletal changes in developing epithelia.

1694

**Mammalian Coronins Regulate Actin Dynamics, Leading Edge Protrusion and Cell Motility**

J. E. Bear, L. Cai, T. Marshall; Cell & Developmental Biology, UNC-Chapel Hill, Chapel Hill, NC

Coronins are actin-binding proteins that regulate cell motility and endocytosis in model organisms. Despite the fact that Coronins are highly conserved across species and have a proven role in motility and endocytosis, the mechanism(s) by which they regulate cellular processes, such as lamellipodial protrusion, remains unclear. Our studies of mammalian Coronins have focused on two representative family members, Coronin 1B and 2A. Coronin 1B is ubiquitously expressed and is localized to protruding leading edge of fibroblasts. This protein interacts directly with the Arp2/3 complex both in vivo and in vitro. The interaction of Coronin 1B and Arp2/3 is regulated by phosphorylation of Ser2 on Coronin 1B. Rat2 fibroblasts expressing the Coronin 1B S2A mutant show enhanced ruffling in response to PMA and increased speed in single cell tracking assays, while the S2D mutant expression attenuates PMA-induced ruffling and slows cell speed. Expression of the S2A mutant partially protects cells from the inhibitory effects of PMA on cell speed. These data demonstrate that Ser2 phosphorylation is responsible for a measurable fraction of PMA's effects on motility. Gene knockdown experiments further indicate that Coronin 1B plays an important role in leading edge protrusion and whole cell motility. Knockdown of Coronin 1B expression can suppress hyper-ruffling phenotypes induced by some, but not all treatments. The second representative Coronin gene, Coronin 2A, is expressed mainly in epithelial and neuronal cell types and is localized along actin stress fibers, but is specifically excluded from the leading edge. Over-expression of Coronin 2A in fibroblasts induces the formation of actin-cofilin rods and reorganizes actin stress fibers into thick bundles that concentrate at the periphery of cells. Together, these data indicate that mammalian Coronins play important roles in actin organization and cell motility.

1695

**Pyk2 Phosphorylation in Osteoclasts is Regulated by the Dynamin GTPase and Src Activity**

A. Bruzzaniti, L. Neff, W. Horne, R. Baron; Dept. of Orthopaedics, Yale University School of Medicine, New Haven, CT

Signaling from the Pyk2-Src-Cbl protein complex is essential for the dynamic regulation of podosomes, which mediate osteoclast (OC) attachment, migration and bone resorption. Accordingly, deletion of any of these genes leads to decreased OC motility, and to osteopetrosis in $Src^{-/-}$ and $Pyk2^{-/-}$ mice. While much is known about Pyk2-Src-Cbl complex assembly, the factors that promote the disassembly of this complex are unknown. We previously showed that dynamin (Dyn) interacts in a Src-sensitive manner with Cbl and localizes to podosomes in OCs. Moreover, overexpression of dominant-negative DynK44A slows actin turnover in podosomes and decreases bone resorption *in vitro*. Since Pyk2 is activated by $Ca^{2+}$ mobilization following integrin engagement and has been implicated in cytoskeletal reorganization, we examined the potential interactions between Dyn and Pyk2. We report that Dyn colocalizes with Pyk2 in the podosome actin ring and forms a complex with Pyk2, independent of the kinase activity of Pyk2. Importantly, phosphorylation of Pyk2 at Tyr402, a residue that is autophosphorylated upon Pyk2 activation and forms the binding site for the Src SH2-domain, is dramatically decreased by the presence of Dyn but not DynK44A. Although Dyn-Pyk2 complex formation was not dependent on Src's adaptor function, Src was required for the Dyn-induced Tyr402 dephosphorylation. Thus, once activated by integrin engagement, Pyk2 is autophosphorylated at Tyr402, recruiting and activating Src at attachment sites. Our study shows that Dyn is also recruited by Pyk2, possibly in the same Src- and Cbl-containing complex. Furthermore, the recruitment of Dyn, in the presence of active Src, leads to the Dyn GTPase-dependent decrease in the phosphorylation of Pyk2 at Tyr402, potentially leading to the dissociation of Src from Pyk2 and/or to decreases in Src and Pyk2 kinase activities which affect podosome turnover and OC function.

1696

**Mammalian Septins Regulate Cell Morphology Through the Adapter Protein NCK**

B. E. Kremer, I. G. Macara; University of Virginia, Charlottesville, VA

Septins are a class of structural GTP-binding proteins found in all eukaryotes except plants. Mammalian septins, of which at least 13 are known, have been proposed to play roles in cell division, secretion, and cytoskeletal regulation. During interphase in cultured cells, Septins 2, -6, and -7 are found in heteromeric filaments that partially align along the actin cytoskeleton. Septin depletion results in the loss of these filaments, as well as disruption of the actin cytoskeleton, defects in cell division, altered focal adhesions, increased rate of cell spreading, and changes in cell morphology. In order to determine the molecular mechanisms underpinning the effects of septin depletion on the actin cytoskeleton and cellular morphology, we stained control- or septin-depleted cells for a variety of proteins involved in cell polarity and actin regulation. We discovered that NCK, a family of two 47-kDa SH2 and SH3 domain-containing adapter proteins, translocates from the cytoplasm to the nucleus following septin depletion. This phenomenon appears to cause the morphological changes following septin depletion. Overexpression of wild type or nuclear export signal (NES)-tagged NCK1 causes knockdown cells to regain their normal elongated morphology. Conversely, nuclear localization signal (NLS)-tagged NCK1 fails to revert the knockdown morphology, and control siRNA cells co-transfected with NLS-NCK1 adopt the rounded morphology characteristic of septin depletion. These results suggest a novel mechanism by which septins regulate the actin cytoskeleton. We are currently investigating the mechanism by which septins regulate NCK localization, as well as the links between NCK localization and the actin cytoskeleton.

1697

**Role of Vinculin in Cytoskeletal Dynamics and Regulation**

L. Jaafar, C. Mierke, D. Paranhos-Zitterbart, P. Kollmannsberger, J. Pauli, W. H. Goldmann, B. Fabry; Center for Medical Physics and Technology, Biophysics Group, Friedrich Alexander Universität, Erlangen-Nürnberg, Erlangen, Germany

The focal adhesion protein vinculin has been implicated in transmembrane mechanical coupling and focal adhesion contact formation [Ezzell et al., *Exp. Cell Res.* **231**:14, 1997]. We and others have demonstrated that F9 mouse embryonic carcinoma cells lacking vinculin (Vin (-/-)) show reduced stress fiber formation and cell stiffness. To test whether vinculin also controls cytoskeletal dynamics, we measured the creep response in F9 Wt, F9 Vin(-/-), and F9 Vin(+/+) rescue cells. Fibronectin-coated superparamagnetic microbeads (diameter 4.5 μm) were bound to the cell surface for 30 min and then pulled in a magnetic field gradient with step forces $F$ ranging from 0.5 to 10 nN. The resulting bead displacement $d$ showed strict power-law behavior over 3 time decades (from $t$ = 0.01 to 10 sec): $d(t) = d_0 t^{\beta}$. Larger $d_0$ implies softer behavior, and a larger power-law exponent β indicates a more viscous (as opposed to elastic), more dynamic behavior [Bursac et al., *Nature Mat* **4**:557, 2005]. At $F$ = 1 nN, F9 Wt cells (n=30) showed $d_0$ = 0.18 μm and β = 0.25 ±0.07; F9 Vin(-/-) cells (n=42) showed $d_0$ = 0.44 μm and β = 0.19 ±0.05; F9 Vin(+/+) rescue

cells (n=29) showed $d_0$ = 0.13 μm and β = 0.27 ±0.05. These results indicate that (i) F9 Vin(-/-) cells are softer, confirming earlier reports that vinculin is important for transmembrane mechanical coupling and (ii) F9 Vin (-/-) cells are less viscous and more elastic, indicating a less dynamic, more stable structure of the cytoskeleton and associated focal adhesions. The findings suggest that vinculin has an important regulatory role in controlling cytoskeletal dynamics. (Financial support for this work came from Deutsche Forschungsgemeinschaft, grant No. Is 25/8-1).

## Actin Dynamics & Assembly II (1698-1716)

1698

### Polymerization of Actin-Nucleotide Complexes

E. L. Weitzke,[1] R. E. Mahaffy,[2] T. D. Pollard,[2] L. M. Loew[1]; [1]Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, CT, [2]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT

The long-term objective of this work is to understand the molecular mechanisms controlling assembly and disassembly of actin filaments at the leading edge of motile cells in terms of the rates of specific biochemical reactions. In this work, the characterization of ATP-, ADP- and ADP-$P_i$-actin monomer and filament behavior is investigated under dynamic and steady state conditions. We aim to quantitatively elucidate nucleation and elongation rate parameters for a wide range of actin concentrations under varying conditions, through a combination of experiment and mathematical modeling. Experimentally, actin polymerization is measured by collecting pyrene fluorescence as a function of time in a fluorimeter. The affect of pyrene labeling on ADP-actin dynamics is investigated under conditions of ADP-actin concentrations close to and far from the critical concentration. Quantitative values for nucleation, elongation and critical concentration for black ADP-actin, actin without pyrene, are obtained through *in vitro* assays and mathematical modeling using the Virtual Cell biological modeling framework. The fraction of pyrene labeled actin affects nucleation curves at concentrations near the critical concentration for ADP-actin, but does not seem to affect elongation rates. Characterization of the three forms of actin is assessed with mathematical modeling and the simulation results are compared with data from *in vitro* polymerization studies involving varying actin concentrations of the three actin forms. ADP-actin polymerizes significantly more slowly than ATP- or ADP-$P_i$-actin, while the latter 2 actin species behave quite similarly. The model permits the derivation of parameters for both the nucleation and elongation phases of these dynamic processes.

1699

### Direct Observation of the Assembly of ATP- and ADP-Actin by Mammalian and Yeast Formins and Profilin

D. R. Kovar,[1,2] E. S. Harris,[3] D. Vavylonis,[4] B. O'Shaughnessy,[4] H. N. Higgs,[3] T. D. Pollard[1]; [1]MCDB, Yale University, New Haven, CT, [2]Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL, [3]Biochemistry, Dartmouth Medical School, Hanover, NH, [4]Chemical Engineering, Columbia University, New York, NY

Formins are large multi-domain proteins required for the assembly of actin filaments found in many cellular structures such as contractile rings, stress fibers and filopodia. Formins nucleate actin filaments and remain processively associated with the fast-growing barbed ends. We have addressed substantial disagreements in the literature by using total internal reflection fluorescence (TIRF) microscopy to observe directly the assembly of individual filaments from ATP- and ADP-actin in the presence of purified constructs containing formin homology domains 1 and 2 (FH1FH2) of two mammalian formins (mDia1 and mDia2) and two yeast formins (Bni1p and Cdc12p). These formins remain processively attached to elongating barbed ends in both the absence and presence of profilin. The four differ in the rapid equilibrium between states that either block or do not block actin subunit addition to the end of the filament. Optimal profilin concentrations increase the rate of barbed-end elongation up to ~5-fold relative to actin alone. ATP-hydrolysis is not required for elongation or processivity. We conclude that although the rates of particular assembly steps vary, these diverse formins are mechanistically similar. In agreement, we have constructed a complete mathematical model that can explain our experimental results and previous crystallographic and biochemical analysis.

1700

### Model for Formin Mediated Elongation of Actin Filaments with Fastened Ends: Filament Torsion, Oscillations and Solution of the Rotation Paradox

T. Shemesh,[1] T. Otomo,[2] M. K. Rosen,[2] A. D. Bershadsky,[3] M. M. Kozlov[1]; [1]Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, [2]Department of Biochemistry, UT Southwesterm Medical Center at Dallas, Dallas, TX, [3]Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel

The FH2 domains of formin family proteins act as processive cappers of actin filaments. Previously suggested stair-stepping mechanisms of processive capping imply that a formin cap rotates persistently in one direction with respect to the filament (Pollard, 2004). This challenges the formin-mediated mechanism of intracellular cable formation and contradicts the *in vitro* results. We suggest a novel scenario of processive capping driven by developing and relaxing torsion elastic stresses. Based on the recent crystal structure of an FH2-actin complex, we propose a second mode of processive capping, the screw mode. Within the screw mode the formin dimer rotates with respect to the actin filament in the direction opposite to that generated by the stair-stepping mode so that a combination of the two modes prevents the persistent torsion strain accumulation. Based on elastic analysis of torsion stresses within an actin filament, we determine an optimal regime of processive capping, whose essence is a periodic switch between the stair- stepping and screw modes. One cycle of this regime consists, in average, of about 12 stair-stepping steps followed by one screw step. We estimate the torsion angle, which can result in actin filament supercoiling and show that within the suggested regime supercoiling of actin filaments is prevented and the filament elastic energy does not exceed feasible values. We analyze coupling between the filament torsion and extension and predict that, upon processive capping by an immobilized formin cap, addition of one actin monomer within the stair-stepping mode should result in filament elongation of about 3.4nm. This excessive elongation is predicted to be balanced by periodic contraction of the filament by about 5nm as a result of screw steps.

1701

### Molecular Regulation of Full-length Formin Proteins

B. L. Goode,[1] S. Maiti,[1] J. B. Moseley,[1] D. Sousa,[1] N. Grigorieff,[2] J. B. Wallingford[3]; [1]Biology, Brandeis University, Waltham, MA,

[2]Biochemistry, Brandeis University, Waltham, MA, [3]Molecular Cell and Developmental Biology, University of Texas, Austin, TX
Formin proteins directly assemble actin filaments through their conserved C-terminal FH2 domains. Within the Diaphanous-related formin (DRF) subfamily, nucleation is thought to be tightly autoregulated by interactions between C-terminal DAD and N-terminal DID domains. Further, it is hypothesized that auto-inhibition is relieved by interactions with Rho-family GTPases. However, full-length formins have not been purified to date to test this mechanism. Here, we developed methods to isolate three full-length DRFs: mouse mDia1 and budding yeast Bni1 and Bnr1, permitting the first direct examination of activity and regulatory mechanisms for full-length formins. In agreement with previous work using fragments (Li and Higgs, 2003), full-length mDia1 is completely autoinhibited. Full-length Bnr1 also is autoinhibited. In contrast, full-length Bni1 exhibits no autoinhibition, despite containing conserved DAD and DID domains. Thus, direct autoinhibition is not a universal DRF regulatory mechanism. Truncation of the DAD fully relieves mDia1 autoinhibition, but RhoA only partially activates full-length mDia1 (10-15% activation), suggesting that alternate mechanisms may control formin activation. To explore this further, we examined the human formin Daam1, which regulates planar cell polarity through the non-canonical Wnt signaling pathway. Daam1 is fully autoinhibited, but Rho, which binds directly to Daam1, fails to relieve autoinhibition. This is consistent with placement of Rho downstream of Daam1 by genetic analyses. Strikingly, Daam1 is fully activated by nanomolar concentrations of its C-terminal ligand Dishevelled, which genetically is placed upstream of Daam1. These data suggest that, in contrast to the prevailing dogma, C-terminal ligands are critical activators of formins. Further, we show that activation involves “opening” and tetramerization of the formin structure, as revealed by analytical gel filtration and electron microscopy and single particle analysis.

1702

**Comparative Analysis of Formin Activities and Regulation**

S. Maiti, J. B. Moseley, B. L. Goode; Rosenstiel Center, Brandeis University, Waltham, MA

Formin proteins directly nucleate actin assembly and processively cap filament barbed ends to allow insertional growth. In all formins examined, the C-terminal FH2 domain mediates these activities on actin. However, due in part to differences in expression source and purification scheme, it has been unclear how the activities of formins from different cell types and organisms compare. Hence, we developed methods in yeast to express and isolate formin proteins from a wide range of species and compare quantitatively their effects on actin assembly. Our comparison includes yeast Bni1 and Bnr1, mouse mDia1 and mDia2, and human Daam1. Thus, the approach we describe should be universally applicable to the purification and analysis of formins from any eukaryote. We show that active FH1-FH2 containing fragments of Bni1, Bnr1, mDia1, mDia2, and Daam1 are each tetrameric, whereas the FH2 domain alone is dimeric. The possible *in vivo* functions of formin tetramerization are presented in models. The FH1-FH2 fragments nucleate actin with variable efficiencies, as high as one actin filament per formin tetramer. We further have used this purification method to isolate soluble full-length mDia1, Bni1, and Bnr1, which have not been possible to purify from bacterial sources. By isolating active formin fragments and full-length formins in parallel, we provide quantitative analyses that address their activities and mechanisms of regulation. We show that three different Diaphanous-related formins (Drfs) are autoinhibited through interactions between their N- and C-termini, yeast Bnr1 and mammalian mDia1 and Daam1. Further, we provide evidence that formins have distinct molecular mechanisms triggering their release from an autoinhibited state.

1703

**Structural Basis for the Autoinhibition and Activation of Diaphanous-Related Formins**

A. Nezami,[1,2] F. Poy,[1] M. J. Eck[1,3]; [1]Dana-Farber Cancer Institute, Boston, MA, [2]Harvard Medical School, Boston, MA, [3]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

Diaphanous-related formins (DRFs) regulate the nucleation and polymerization of unbranched actin filaments. DRFs are regulated by the intramolecular binding of the C-terminus Diaphanous-autoinhibitory domain (DAD) to the N-terminus DAD-interacting domain (DID). This inhibition is relieved by Rho-GTP binding to the N-terminal GTPase binding domain (GBD). We have determined high resolution crystal structures of the DID region of mDia1, both alone and in complex with the inhibitory DAD domain. Additionally we have determined the structure of a large N-terminal fragment of mDia1 containing the DID, dimerization, and coiled coil domains. The DAD domain adopts a mostly helical conformation and binds via extensive hydrophobic and polar interactions to a highly conserved groove on the DID domain. Comparison of these structures with the previously reported structure of the activated mDia1 N-terminus in complex with a Rho GTPase reveals that release of the inhibitory DAD interaction is effected mainly by Rho-induced conformational changes in the mDia1 N-terminus, and to a lesser degree by direct steric clash between Rho on the DAD sequence. Calorimetric analyses of the interaction of N-terminal fragments of mDia1 with C-terminal fragments including either or both of the FH2 and DAD domains indicate that the DAD/DID interaction revealed in the crystal structure is the complete autoinhibitory contact.

1704

**Studies of Diaphanous-related Formin Autoregulation Yields Novel Activated Variants of the mDia2 Sufficient to Induce Filopodia Assembly**

H. A. Holman, B. J. Wallar, A. S. Alberts; Cell Structure & Signal Integration, Van Andel Research Insitute, Grand Rapids, MI

Rho GTPases activate the mammalian Diaphanous-related (mDia) formins by disrupting an intramolecular autoinhibition mechanism maintained by the association of conserved Dia-inhibitory (DID) and the C-terminal Dia-autoregulatory (DAD) domains that flank the formin homology-2 (FH2) domain. The loss of autoinhibition allows the FH2 domain to nucleate and elongate non-branched actin filaments. In this study, we have defined additional regions with DAD that are required for DID binding. Versions of mDia2 that lack the N-terminal autoinhibitory domains, including all or part of the GTPase-binding domain and/or DID induce the formation of thin actin filaments resembling stress fibers. In contrast, expression of DAD-truncated mDia2 or DAD-mutated versions of mDia2 that block binding to DID - leaving the GTPase-binding domain intact - leads to the generation of filopodia in the absence of other stimulation. Time-lapse microscopy shows these activated variants of mDia2 at the tips of elongating actin structures. These findings are consistent with the observation that mDia2 is a critical component of actin filament assembly at the cell cortex.

1705

**RhoB and the Mammalian Diaphanous-related Formin mDia2 Govern Actin Dynamics on Endosomes Necessary for Trafficking and Vesicle Fusion**

B. J. Wallar, J. Peng, K. A. Collins, K. M. Eisenmann, A. S. Alberts; Cell Structure & Signal Integration, Van Andel Research Institute, Grand Rapids, MI

Mammalian Diaphanous-related (mDia) formins nucleate and elongate actin filaments following binding and activation by Rho GTPases. While all three mDia proteins (mDia1/2/3) have been shown to localize to endosomes, the role of GTPase-activated formins in actin assembly on endocytic vesicles has not been fully characterized. Some models have been proposed based on the observation that constitutively activated Rho GTPase and mDia proteins trigger the assembly of stress fiber-like actin filaments that act as conduits for vesicle transport. Here, in a study of a functional relationship between RhoB and mDia2, we show that: 1) RhoB and mDia2 interact on endosomes; 2) GTPase activity - the ability to hydrolyze GTP to GDP - is required for the ability of RhoB to govern endosome dynamics; and 3) actin treadmilling controlled by RhoB and mDia2 is necessary for vesicle trafficking as well as for the process of vesicle fusion. These studies further show that Rho GTPases significantly influence the activity of mDia family formins to drive cellular membrane dynamics through the process of actin treadmilling.

1706

**Actin Nucleation by Cappuccino-family Formins is Regulated by Spir-family Proteins**

M. E. Quinlan,[1] S. Hilgert,[2] E. Kerkhoff,[2] R. D. Mullins[1]; [1]Cell and Molecular Pharmacology, UCSF, San Francisco, CA, [2]Institut fur Medizinische Strahlenkunde und Zellforschung, Universitat Wurzburg, Wurzburg, Germany

The actin cytoskeleton is essential for many cellular functions including shape determination, intracellular trafficking, and locomotion; formin-family proteins contruct many of the actin networks involved in these functions. Phylogenetic analysis identifies seven classes of actin-nucleating formins in metazoa. Most of these classes contain a conserved regulatory domain (Diaphanous Autoregulatory Domain, DAD), which inhibits nucleation activity in the absence of an upstream activator. Formins related to the *Drosophila* Cappuccino protein, however, lack the DAD domain and their regulation is poorly understood. We find that a novel domain originally discovered in Spir-family proteins (the kinase non-catalytic C-lobe domain, or KIND), binds the FH2 domain of Cappuccino-family formins with submicromolar affinity. The interaction directs localization of Cappuccino in tissue culture cells and potently inhibits its actin nucleation activity *in vitro*. The interaction is conserved from *Drosophila* to mammals and is highly specific, as we find that Spir proteins do not interact with other classes of formins (e.g. the Diaphanous-family proteins). Spir proteins are themselves actin nucleation factors that operate by a mechanism distinct from those of the formins or the Arp2/3 complex. Spir assembles actin nuclei using four monomer-binding WH2 domains and an additional motif, conserved among Spir proteins. We find that each WH2 domain plays a distinct role in the nucleation reaction and that the identity and position of each WH2 domain is important for maximal activity. Previous work has shown that Spir and Cappuccino are both required for axis specification in *Drosophila* oocytes and embryos and that mutation of either protein produces the same phenotype. Spir and Cappuccino homologs in mammals also have identical tissue distributions in both embryos and adults. Together our data indicate that Spir and Cappuccino interact directly and collaborate to construct essential cytoskeletal structures required for development.

1707

**Model of Formin-Associated Actin Filament Elongation and the Role of Profilin**

D. Vavylonis,[1,2] D. R. Kovar,[2] B. O'Shaughnessy,[1] T. D. Pollard[2,3,4]; [1]Chemical Engineering, Columbia University, New York, NY, [2]Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, [3]Cell Biology, Yale University, New Haven, CT, [4]Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

Formins control the kinetics of actin filament assembly by remaining processively attached at the growing actin filament barbed ends. The formin FH2 domain associates with the barbed end while FH1 interacts with profilin and is thought to mediate profilin-actin polymerization. We combined total internal reflection microscopy (Kovar et al., separate submission) with theoretical modeling to study the dependence of the rate of actin filament elongation on profilin and actin concentrations for four different FH1-FH2 constructs (mouse mDia1, mouse mDia2, budding yeast Bni1p, and fission yeast Cdc12p). Taking into account earlier crystallographic and biochemical data we constructed a kinetic model which explains the main experimental trends. The model is based on a conserved transfer mechanism of profilin-actin from FH1 to the barbed end, gated by FH2 as described by a gating parameter, p. Differences between formins are attributed to different parameter values. The main experimental observation, namely the existence of an optimal profilin concentration where elongation rate is a maximum, is another central conclusion of the model. We rationalize (i) how filaments associated with mDia1 elongate more rapidly than formin-free filaments due to the the large number of profilin binding sites and the large gating parameter (p~1) characterizing mDia1 and (ii) how filaments associated with formins whose p values are small, such as mDia2, elongate slowly in the absence of profilin but more rapidly in the presence of profilin. In all cases high enough profilin concentrations suppress elongation rates; in the model this is attributed largely to the saturation of FH1 by free profilin. Consistent with our ADP-actin experiments, the proposed mechanism does not require ATP hydrolysis though we cannot exclude the possibility that formin translocation accelerates ATP hydrolysis for ATP-actin.

1708

**APC2 and Diaphanous Function Together to Organize Actin in *Drosophila* Embryos**

R. L. Webb, J. S. Weinberg, B. M. McCartney; Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

Coordinated assembly and disassembly of actin filaments is a hallmark of cell migration and the orchestrated cell shape changes that occur during morphogenesis. Adenomatous polyposis coli (APC) family proteins associate with actin and microtubules influencing cell migration via actin through IQGAP and a RacGEF, and by promoting microtubule stability downstream of Rho with the formin Diaphanous and the microtubule + TIP protein, EB1. Here we report a novel role for APC2 and Diaphanous in organizing actin in Drosophila syncytial embryos. We have shown previously that Drosophila APC2 localizes to actin structures and the phenotype of mutant syncytial embryos suggested a role for APC2, Drosophila ß-catenin and GSK3ß, in tethering cortical actin to microtubules. To test this hypothesis, we examined embryos null for *APC2* function and observed defects in the actin rings and furrows that develop during syncytial mitoses to separate adjacent mitotic figures. This suggests a role

for APC2 in the assembly or stability of actin-based structures. Diaphanous also plays a role in actin ring and furrow formation in the syncytial embryo. We found that APC2 and Diaphanous colocalize and coimmunoprecipitate from syncytial embryos, suggesting that these proteins form a complex. Further, we showed that reduction of *diaphanous* in *APC2* mutant syncytial embryos enhances the severity of actin defects compared to those in *APC2* mutant embryos alone. To complement the analysis of fixed embryos, we are using live imaging to assess dynamic actin behavior in wild type and mutant embryos. We have observed a variety of actin defects in *APC2* mutant and *dia/+; APC2* mutant embryos consistent with a role for these proteins in actin assembly and stability. We are currently assessing the role of APC2 in the RhoGEF2 and Rho1 regulated actin organization function of Diaphanous in Drosophila.

1709

**Aip1 Accelerates the Turnover of Actin Networks *in vivo* by a Mechanism of Capping ADF/cofilin-severed Actin Filaments**

K. Okada,[1] H. Ravi,[2] E. M. Smith,[1] B. L. Goode[1]; [1]Rosenstiel Basic Medical Research Center, Brandeis University, Waltham, MA, [2]Department of Biochemistry, Brandeis University, Waltham, MA

Actin interacting protein (Aip1) is a highly conserved component of the actin cytoskeleton that has been linked functionally to ADF/cofilin, but its mechanism of action and cellular function has remained poorly understood. Here, we demonstrate that Aip1 and cofilin accelerate the turnover of both actin patches and actin cables in *S. cerevisiae*. Both of these actin structures are highly dynamic, and loss of cofilin and Aip1 functions results in greatly reduced dynamics and loss of cellular functions. To dissect and understand better the Aip1 cellular mechanism, we integrated mutations at 31 conserved residues on its surface and performed genetic, two-hybrid, and biochemical analyses of mutant effects. We identify two distinct and well-separated surfaces on Aip1 that are important for its functions *in vivo* and mediate direct binding to F-actin. Mutations at these sites disrupt Aip1-induced capping and net depolymerization of ADF/cofilin-severed filaments *in vitro*. These same mutations lead to loss of Aip1 localization, thickened actin cables, and genetic interactions with *cof1-22 in vivo*. In addition, these *aip1* mutations reduce rates of actin cable and patch turnover in living cells. Together, these data suggest that the primary cellular function of Aip1 is to accelerate actin turnover by capping cofilin-severed filaments. They also provide the first mechanistic insights into the regulation of yeast actin cable turnover. Our results suggest that Aip1 may have a conserved role in switching the effects of cofilin, from severing filaments to promote new growth in the absence of Aip1 to inducing net depolymerization and turnover of filaments in the presence of Aip1.

1710

**Cofilin-induced Switch from Intramolecular to Intermolecular Cross-linking in Skeletal F-Actin**

M. Phan, Y. Yang, Y. Xie, S. Benchaar, R. R. Ogorzalek Loo, J. A. Loo, E. Reisler; Chemistry & Biochemistry, UCLA, Los Angeles, CA

Actin is believed to assume different conformational states in the monomer and the polymer forms. In turn, these states are altered upon binding ADF/cofilin to actin. Such conformational rearrangements in actin can be identified by using chemical cross-linking methods. Benzophenone-4-maleimide (BPM), an 8Å cross-linking reagent, can be attached to Cys374 of skeletal actin and then photo-actived to cross-link within actin. We confirmed that intramolecular cross-linking with this reagent occurs in F-actin but not in G-actin. Minor intermolecular cross-linking in F-actin has been also detected. Using mass spectrometry methods, the intramolecular cross-link was mapped to Cys374 and Asp11; the intermolecular cross-link was mapped to Cys374 and Met44 on adjacent protomers within the same filament strand. The abundant cross-linking between Cys374 and Asp11 in F-actin, despite their 17.2 Å separation in both G-actin and F-actin (Holmes 1990), provides experimental evidence for a polymerization driven structural change in actin. The binding of cofilin to F-actin abolishes the intramolecular cross-linking and alters the conformation of actin to favor the intermolecular Cys374-Met44 reaction. This intermolecular cross-link locks actin in a conformational state with increased affinity for cofilin. Cofilin appears to be "trapped" on the cross-linked protomers and does not easily dissociate from the actin, even under high salt conditions. Thus, BPM cross-linking from Cys374 to different sites on actin maps some of the conformational changes that occur on actin upon its polymerization and the binding of cofilin.

1711

**Actin Filament Flexibility and Severing by Cofilin and Gelsolin**

D. A. Pavlov, E. Reisler; Chemistry and Biochemistry, UCLA, Los Angeles, CA

Previous studies using differential scanning calorimetry (DSC) showed dual effects of cofilin on F-actin: a stabilization of the occupied protomers and cooperative destabilization of the unoccupied parts of the filament at subsaturating cofilin concentrations. Thus, filaments fully decorated by cofilin are more stable. *In vitro* severing experiments using fluorescent microscopy showed that filaments tethered to the glass surface *via* barbed ends or loosely attached at low densities of heavy meromyosin (HMM) were severed at lower rates, while multipoint-tethered filaments were severed by cofilin at higher rates. Cofilin-induced structural strain in actin filaments facilitates their severing; this strain is dissipated when the number of attachment points is reduced, decreasing cofilin's severing activity. In this study, we contrast the effects of gelsolin on filament stability (in DSC) and the *in vitro* severing with those of cofilin. Differential scanning calorimetry (DSC) showed that monomeric actin was stabilized upon gelsolin, gelsolin segment 1, or cofilin binding. DSC of F-actin showed different structural effects of gelsolin and cofilin binding to F-actin. Actin polymerization caused an increase in $T_{max}$ (8ºC) in DSC experiments. Actin copolymerized with gelsolin (at mole ratios from 3000:1 to 100:1) denatured at lower temperature than intact F-actin; the effect increased with greater amount of incorporated gelsolin, but was detected even at 1:3000 gelsolin to actin ratio. In electron microscopy observations, actin filaments copolymerized with bigger amounts of gelsolin were generally shorter. *In vitro* severing experiments showed that, in contrast to cofilin, increased tethering inhibited actin severing by gelsolin. These results reveal F-actin structure and dynamics dependent differences in filament severing by different proteins.

1713

**Two Region-dependent Functions of PAK: Control of Polymerization by Cofilin Inhibition in the Lamellipodium and of Contraction by Myosin II Activation in the Lamella**

V. D. Delorme,[1] M. Machacek,[2] C. Waterman-Storer,[2] G. Danuser,[2] G. Bokoch[1]; [1]Immunology, The Scripps Research Institute, La Jolla, CA, [2]Cell Biology, The Scripps Research Institute, La Jolla, CA

p21-activated kinases (PAKs) are downstream effectors of Rho-family GTPases that signal to the actin cytoskeleton assembly and actin contraction

machineries. The molecular mechanisms involved in this dual action of PAK remain to be defined. To assess the function of PAK1 at the cell leading edge, we selectively perturbed components of the actin assembly signaling pathway Rac1-PAK1-LIMK-cofilin and the contraction pathway Rac1-PAK1-MLCK/myosinII, respectively. We measured the changes in actin dynamics using quantitative Fluorescent Speckle Microscopy to obtain the actin network velocity (retrograde flow) and assembly and disassembly rates in two regions: the lamellipodium, which is a 1 µm wide region adjacent to the leading edge, and the lamella, which extends from the leading edge several microns into the cell. We tracked the leading edge to assess the changes in rates of protrusion and retraction. We observed the distribution of markers of the lamellipodium (Arp3, cofilin) and the lamellum (tropomyosin, myosin, paxillin). We show that inhibition of PAK1 blocks active RacQ61L-induced actin and protrusion dynamics: inhibition reduces the width of the lamellipodium, possibly by increasing cofilin dephosphorylation and activity. PAK1 inhibition is further associated with a dramatic decrease in the rate of actin retrograde flow in the lamellum. This effect appears to be due to regulation of myosinII-dependent contraction. In contrast, direct enhancement of cofilin activity downstream of PAK1 (via inactive LIMK or non-phosphorylatable cofilin mutants) increases the rate of actin retrograde flow in the lamella. The distribution of biochemical markers indicative of the two regions is also modified. Our results indicate a region-dependent functionality of PAK1: we conclude that PAK1 promotes turnover of the actin network in the lamellipodium and activates myosin II contractility in the lamella.

1714

### The Two *Caenorhabditis elegans* Actin Depolymerizing Factor/Cofilin Proteins Differently Enhance Actin Filament Severing and Depolymerization

S. Yamashiro,[1] K. Mohri,[1,2] S. Ono[1]; [1]Pathology, Emory University, Atlanta, GA, [2]Department of Biophysics, Kyoto University, Kyoto, Japan

Actin depolymerizing factor (ADF)/cofilin is a conserved family of actin-regulatory proteins among eukaryotes that are critical for rapid turnover of actin filaments. Multicellular organisms express multiple ADF/cofilin isoforms in a tissue specific manner, and they have quantitatively different actin-regulatory activities. The *Caenorhabditis elegans unc-60* gene encodes two ADF/cofilin family proteins which are designated as UNC-60A and UNC-60B. Our previous studies suggested that UNC-60A and UNC-60B are functionally differentiated ADF/cofilin isoforms. UNC-60A is expressed in various tissues and required for early embryogenesis, whereas UNC-60B is specifically expressed in body wall muscle and required for myofibril assembly. In steady state assays in vitro UNC-60A causes greater net depolymerization of actin filaments than UNC-60B. Here, we further compared their differences in activities to sever and depolymerize actin filaments using actins from rabbit muscle and *C. elegans*. The non-muscle isoform UNC-60A had greater activities to cause net depolymerization and inhibit polymerization than the muscle specific UNC-60B. Surprisingly, UNC-60B showed much stronger severing activity than UNC-60A. Moreover, UNC-60B induced much faster pointed-end depolymerization of rabbit muscle actin than UNC-60A, while UNC-60A caused slightly faster depolymerization of *C. elegans* actin than UNC-60B. These results suggest that the muscle specific UNC-60B is kinetically more efficient in enhancing actin turnover than the non-muscle isoform UNC-60A, while UNC-60A is suitable for maintaining higher concentrations of monomeric actin. These functional differences might be specifically adapted for different actin dynamics in muscle and non-muscle cells.

1715

### Vav1 Regulates the Activity and Localization of Cofilin-phosphatase Slingshot1L

H. Matsuoka, K. Ohashi, K. Mizuno; Department of Biomolecular Sciences, Graduate School of Life Sciences Tohoku University, Sendai, Japan

Actin cytoskeletal dynamics and reorganization play pivotal roles in a variety of cell activities, including migration, morphological change, polarity formation and cytokinesis. Cofilin, a key regulator of actin filament dynamics, is inactivated by phosphorylation at Ser-3 by LIM-kinase 1/2 (LIMK1/2) and is reactivated by dephosphorylation by a family of protein phosphatases, termed Slingshot1/2/3 (SSH1/2/3). As for the mechanism of regulation of LIMK activity, it is proposed that both Rho-ROCK and Rac-PAK signaling pathways activate LIMK1. However, the mechanism for regulating SSH activation and cofilin dephosphorylation is not well understood. To elucidate the mechanism of regulation and function of SSH1L, we employed proteomic approach comprising tandem affinity purification (TAP) method. Using MALDI-TOF/MS analysis, we identified Vav1 as one of the SSH1L-associated proteins. Vav1 is a guanine nucleotide exchange factor for Rho family GTPases (Rho/Rac). The cell-free and co-precipitation analyses revealed that SSH1L is inactive when it associates with Vav1. Furthermore, microscopic analysis and F-actin co-sedimentation assays suggested that Vav1 controls the localization of SSH1L in response to an extracellular stimulus. Based on these results, we suggest the role of Vav1 in the regulation of SSH1L activity as follows: an extracellular stimulus leads to the activation and membrane translocation of Vav1, which results in the recruitment of SSH1L to the plasma membrane, where SSH1L is released from Vav1 to be activated.

1716

### Molecular Motors in Cells: A Rapid Switch of Biopolymer Organization

D. M. Smith,[1] F. Ziebert,[2] W. Zimmermann,[2] J. A. Käs[1]; [1]Institute for Soft Matter Physics, University of Leipzig, Leipzig, Germany, [2]Physikalisches Institut, Universität Bayreuth, Bayreuth, Germany

All eukaryotic cells rely on self-assembly of protein filaments to form an intracellular cytoskeleton. The necessity of motility and reaction to stimuli additionally requires pathways that reversibly change cytoskeletal organization. While temperature-driven disordering is, from the viewpoint of physics, the most obvious method for dissolving complex cellular structures, this approach would exceed the physiologically viable temperature range. This motif is exemplified in the de-hybridization of DNA, which is temperature-induced in PCR, but achieved by molecular motors in cells. We report another fundamental mechanism whereby changes in the activity of Myosin II induce order-disorder transitions in reconstituted cytoskeletal actin-myosin networks. Bulk activity of the motors, which causes sliding of individual filaments (D. Humphrey et al, *Nature* 2002), maintains a dynamically disordered network. During depletion of the ATP supply, an increasing fraction of molecular motors becomes inactive, crosslinking actin filaments to small clusters. The remaining active motors combined with continually increasing cross-linking foster further growth of these clusters, resulting in a variety of ordered macro-molecular structures such as asters, networks resembling neuronal architectures, and condensed super-precipitates. Experiments with photo-activated motors demonstrate the quick reversible restoration of the disordered state. This nonequilibrium pathway to switch between order and disorder is much faster than any structural changes driven by Brownian motion in thermodynamic equilibrium. This ability for rapid, isothermal motor-induced transitions between different degrees of self-organization indicates that molecular motors, in general, may substantially contribute to dynamic cellular organization.

## Muscle: Biochemistry & Cell Biology I (1717-1733)

1717

### Immaturity of Microtubule Networks in Alpha B-crystallin Antisense C2C12 Mouse Myoblast Cells

T. Sakurai, Y. Atomi; Department of Life Sciences, The Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan

Alpha B-Crystallin, one of the small heat shock proteins, has chaperon activity and may protect cytoskeletons under stress conditions and decreases during early skeletal muscle atrophy but not under stretched conditions. We have previously demonstrated interactions between tubulin/microtubules and alpha B-crystallin in cell culture extracts and rat skeletal muscle extracts using immunopresipitation and microtubule sedimentation assay. These data imply an interaction between alpha B-crystallin and tubulin/microtubules in muscle tissues. To investigate a precise role of alpha B-crystallin in vitro, C2C12 mouse myoblast cells transformed with a sense (C2C12SE) or antisense strand (C2C12AS) of alpha B-crystallin cDNA were cultured and observed the microtubule networks by immunofluorescence study. The expression levels of alpha B-crystallin were about 10-fold higher in C2C12SE cells and low in C2C12AS cells. We observed that C2C12WT and C2C12SE cells fused well. Especially, C2C12SE cells formed thick myotube. On the contrary, C2C12AS cells only lined up but could not differentiate into myotube at all. Hoechst staining revealed C2C12WT and C2C12SE cells differentiated into multinucleated myotube, but C2C12AS did not. The microtubule networks in C2C12SE cells developed well, and the dominant direction of the microtubules was longitudinal. On the other hand, the microtubule networks in C2C12AS were thin and spread rather radially than longitudinally. Though alpha B-crystallin may have other rolls because C2C12AS was observed to be too hard to fuse into myotube, these observations imply that alpha B-crystallin is essential for the formation of dense microtubule networks and may keep microtubule reorganization during differentiation. Our previous work that the decrease of alpha B-crystallin increased the rate of nocodazole-induced depolymerization of microtunbules also supports an important roll of alpha B-crystallin for microtubule network maintenance.

1718

### Non-muscle Myosin 2A and 2B are Essential for Myoblast Alignment and Fusion

N. Swailes, P. J. Knight, M. Peckham; University of Leeds, Leeds, United Kingdom

Mononucleated myoblasts form aligned groups of cells prior to fusion, in which the cells have a characteristic bipolar shape, in which the majority of the actin cytoskeleton is organised into a subplasmalemma network, 500nm thick, with mixed polarity (Swailes et al., 2004). Two non-muscle myosin isoforms (2A and 2B) are associated with this network and our objective here was to determine whether they function differently in the formation of aligned groups of myoblasts. Using immuno-electron microscopy we found that the distribution of these two myosins overlaps, but myosin 2A is more tightly associated with the actin network, with the majority of myosin 2A lying within 500nm of the plasma membrane, whereas only 50% of myosin 2B lies within this region. Using an antisense oligonucleotide treatment to knock down the expression levels of either myosin 2A or 2B, we found that both treatments interfered with formation of aligned groups and fusion, but in a different way. 2A knockdown prevented the formation of bipolar cells, and cell-cell adhesion. In contrast 2B knockdown resulted in highly elongated cells, that were double the length of untreated or control cells (treated with sense or scrambled oligonucleotides). Live cell imaging confirmed that this elongation was the result of poor cell retraction. Therefore, both of these myosins play an essential but different role in cell-cell alignment prior to fusion in myoblasts.

1719

### In Vivo Interactions of MyoD, Id1 and E2A Proteins Determined by Acceptor Photobleaching Fluorescence Resonance Energy Transfer

J. Lingbeck; Washington University School of Medicine, St. Louis, MO

MyoD, a skeletal muscle transcription factor, is rapidly degraded by the ubiquitin-proteasome system in both the cell nucleus and the cytoplasm. MyoD interacts with ubiquitously expressed E2A or Id proteins to activate or inhibit transcription, respectively. Furthermore, MyoD has been shown to modulate the ubiquitin-mediated degradation of Id1 and E2A proteins (E12 and E47). E12 and E47 also reduce the rates of degradation of MyoD and Id1 via the ubiquitin-proteasome system, however, the molecular mechanisms governing these events are not clear but are thought to occur via heterodimer formation. Fluorescence resonance energy transfer (FRET) which measures the energy transfer from a donor molecule to an acceptor molecule within 100 Å is a powerful technique for evaluation of protein-protein interactions *in vivo*. Using acceptor photobleaching FRET and chimeric proteins composed of MyoD, Id1, E12, E47, $E12^{NLS}$, or $MyoD^{NLS}$ and either CFP or YFP we show that MyoD, Id1, E12 and E47 each form homodimers within the cell. Furthermore, we show 1) MyoD interacts directly with Id1, 2) E12 and E47 each dimerize with MyoD and with Id1 and 3) E12 interacts with Id1, but not MyoD in the cytoplasm. The FRET results further show stronger binding of Id1 to E12 or to E47 than to MyoD which supports the notion that regulation of MyoD occurs by the sequestration of E2A proteins by Id1 both in the nucleus and in the cytoplasm. It also suggests that heterodimerization is likely responsible for the decreases in the degradation rates of these proteins via the ubiquitin-proteasome system.

1720

### MRF4 is Upstream of MyoD, Myf5, Myogenin in Transactivation Hierarchy during Myogenic Differentiation of Bovine Adult Myoblasts

Y. Matsubara,[1] Y. Hondo,[1] A. Yamaguchi,[1] M. Miyamoto,[1] S. Muroya,[2] K. Maruyama[1]; [1]Department of Life Science, Meiji University, Kawasaki, Japan, [2]Department of Animal Products, National Institute of Livestock and Grassland Science, Ibaraki, Japan

Myogenic regulatory factors (MRFs: MyoD, Myf5, myogenin, and MRF4) control the differentiation of muscle lineage cooperatively. However, the hierarchy of their transactivation and their roles in the lineage determination and differentiation have not been defined. In this study, the interaction among MRFs was investigated in bovine adult myoblasts using RNA interference with a hypothesis that MRF4 was positioned upstream of MyoD, Myf5, and myogenin in transactivation hierarchy. Adult myoblasts were isolated from bovine *Musculus Biceps femoris* and purified to contain desmin-positive cells at more than 95%. Small interfering RNA against respective MRFs: siMyoD, siMyf5, simyogenin, and siMRF4 were designed and custom-synthesized. After the myoblasts were transfected with a single or a combination of siRNAs and differentiation was induced by reducing the amount of FBS in the medium, the MRF expression was detected by real-time reverse-transcription polymerase chain reaction two days later. Myogenic differentiation was analyzed by the monoclonal antibody staining for MyHC and the nuclear staining with Hoechst33258 six days later. The expression of MyoD, Myf5, myogenin, and MRF4 was reduced to 56.4%, 61.5%, 25.7%, and 33.1%,

respectively, by the respective siRNAs. The siMRF4 reduced all MRFs expression. The siMyoD reduced Myf5 and myogenin expression, while increasing MRF4 expression. The simyogenin reduced MyoD and Myf5 expression without affecting MRF4 expression. The siMyf5 reduced only myogenin expression. When myoblasts were transfected with siMyoD and siMyf5, the expression of all but MRF4 was reduced. The fusion index (percentage of nuclear number in the MyHC-positive cell/total nuclear number) was reduced by siMyoD or siMRF4. The siMyoD drastically suppressed the myotube formation. The polynuclear index (the number of nuclei in the MyHC-positive cells) was elevated by siMyf5 and reduced by simyogenin.

1721

**Stage-dependent Protein Degradation via the Ubiquitin Proteasome Pathway Regulates E2A Protein Levels during Muscle Differentiation**

L. Sun,[1] J. S. Trausch-Azar,[1] A. Ciechanover,[2] A. L. Schwartz[1]; [1]Department of Pediatrics and Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO, [2]Department of Biochemistry and Rappaport Institute for Research in Medical Sciences, Technion-Israel Institute for Technology, Haifa, Israel

The E2A proteins, (i.e. E12 and E47, the two splice variants encoded by the *E2A* gene), are basic helix-loop-helix transcription factors that regulate differentiation and proliferation in many cell types. In muscle cells, the E2A proteins form heterodimers with muscle regulatory factors such as MyoD, which then bind to DNA and up-regulate the transcription of target genes. Hence, regulation of the E2A protein level is likely important for muscle differentiation. In C2C12 cells we show that endogenous E2A proteins are primarily localized in the nucleus in both myoblasts and myotubes, and appear to be degraded by the ubiquitin proteasome system as the proteasome inhibitor, MG132, stabilized E12 and E47 in both myoblasts and myotubes. Cellular abundance of E2A proteins is down regulated during the differentiation from myoblasts to myotubes. The observed reduction in E2A protein levels appears to result from a change in the rate of protein degradation rather than synthesis. Ubiquitin-proteasome-mediated E2A protein degradation is dependent on the muscle differentiation state, with t1/2 ~ 2h in proliferating myoblasts versus t1/2 ~ 7h in differentiated myotubes. No change in E2A mRNA abundance was detected via RT-PCR nor was a change in E2A protein synthesis detected in myoblasts versus myotubes. These results support an important role for E2A protein in the regulation of muscle differentiation, and have implications for the involvement of ubiquitin-proteasome-mediated protein degradation in muscle development under abnormal and pathological conditions.

1722

**Indirect Flight Muscles (ifm) of *Drosophila*: A Model for Human Nemaline Myopathy**

V. Kumar,[1,2] J. C. Sparrow,[2] M. Peckham[1]; [1]The School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom, [2]Biology, University of York, York, United Kingdom

The nemaline myopathies (NM) are a group of human neuromuscular disorders characterized by muscle weakness and the presence of nemaline bodies/rods in skeletal muscle fibres. They are caused by mutations in thin filament proteins (nebulin, actin, troponin and tropomyosin), but actin mutations are the most common. Over 100 mutations in the human skeletal α-actin gene, *ACTA1*, have been identified, of which most are dominant and occur in families with no previous history of the disease (reviewed by Sparrow *et al*., 2003). We investigated how mutations in skeletal actin cause NM using Drosophila indirect flight muscle (IFM) as a model system. IFM specific actin (ACT88F) is highly homologous (93% conserved) to human skeletal α-actin. Using p-element transformation, transgenic flies were generated for three different mutations: G15R, V163L and V163M, that were either heterozygous or homozygous for the mutation. We found that these mutations resulted in a range of phenotypic changes similar to those seen in humans with nemaline myopathy, including variable sarcomere lengths and 'zebra bodies', confirming that the Drosophila IFM system is a good model for this disease. Results from immuno-staining and electron microscopy show that assembly of thin filaments is abnormal during muscle development, and that the filament lengths are highly variable. This suggests that the major defect resulting from the NM mutations is mis-regulation of filament length.

1723

**Domain Function and Genetic Analysis of the *C elegans* ALP/Enigma Gene, *alp-1***

H. Han,[1,2] C. R. McKeown,[2] M. C. Beckerle[1,2]; [1]Oncological Sciences, University of Utah, Salt Lake City, UT, [2]Huntsman Cancer Institute, Salt Lake City, UT

ALP-Enigma proteins are prominently expressed in muscle and display one N-terminal PDZ domain and one or three C-terminal LIM domains. Targeted gene disruption studies in the mouse have suggested that ALP-Enigma proteins are critically important for muscle stability, and deficiencies in this family are associated with muscle disease. To further study these proteins, we identified a single genetic locus, *T11B7.4*, which encodes the entire ALP-Enigma protein family in *Caenorhabditis elegans*. The *alp-1* gene encodes four transcripts that result from alternative splicing. *alp-1a* encodes an ALP homologue, and *alp-1b*, *c*, and *d*, encode Enigma-like proteins. ALP-Enigma proteins are localized at dense bodies and dense plaques, two actin anchorage sites in *C. elegans* muscle. To understand the function of the shared PDZ domain, we generated transgenic worms expressing the ALP-1 PDZ domain under the control of a heat-shock promoter. Our data demonstrate that the ALP-1 PDZ domain is sufficient for localization to dense bodies within muscle cells, but not to dense plaques. To explore ALP-Enigma function *in vivo*, we have characterized two *alp-1* deletion alleles (generated by the *C. elegans* Gene Knockout Consortium and National Bioresource Project). The more severe *alp-1(tm1137)* mutant is predicted to encode a protein in which LIM1 is deleted and premature translational termination occurs. By Western immunoblot analysis, we fail to detect ALP-1A, B or D protein products in the *alp-1(tm1137)*. Although our current reagents cannot detect the expression of ALP-1C, the nature of the *alp-1(tm1137)* mutation suggests that ALP-1C would also be affected by the deletion. Phenotypic analysis demonstrates that the *alp-1* mutants display pharyngeal pumping, locomotion and brood size that are indistinguishable from wild-type. Thus, in contrast with vertebrate systems, muscle function is not compromised in the *C. elegans alp-1* mutants analyzed to date.

1724

**Visualization of Ectopic Calcification in mdx Mouse Skeletal Muscle 2**

N. Kikkawa,[1] M. Shiozuka,[1] T. Kogure,[2] R. Matsuda[1]; [1]The Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan, [2]The Graduate School of Science, The University of Tokyo, Tokyo, Japan

It has been demonstrated that osteogenic differentiation of skeletal muscle cells can be induced by osteogenic factors, such as bone morphogenetic protein-2 (BMP-2), both in vitro and in vivo. One example of spontaneous ectopic calcification was seen in skeletal muscle of mdx mouse, a model of Duchenne muscular dystrophy (DMD). Ectopic calcification was observed by using high-resolution X-ray microtomography Skyscan 1074, which gives resolution of 22um. The compound of this ectopic calcification was revealed to be hydroxyapatite by electron dispersive X-ray spectrometry and X-ray diffraction. Ectopic calcification tended to increase with age in mdx mouse, while no ectopic calcification was observed in skeletal muscle of B10 mouse, which possesses normal dystrophin gene and was used as a negative control. Alkaline phosphatase activity of mdx mouse skeletal muscle was higher than in B10 mouse, but the region of high activity did not overwrap with the calcification. Correlations between muscle degeneration and ectopic calcification were studied by intraperitoneal injection of calcein, tetracycline and Evans blue. The former two were used to monitor calcification, and the last one as a marker of muscle degeneration. Calcified regions and Evans blue positive regions did not coincide, suggesting that calcification does not occur immediately after muscle degeneration.

1725

**Alteration of FHL1 & FHL3 Protein Levels and Localisation in a Congenital Muscle Disease and Hypertrophy**

M. T. Nguyen,[1] J. E. Joya,[1] C. A. Mitchell[2]; [1]Muscle Development Unit, Children's Medical Research Institute, Sydney, Australia, [2]Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Australia

Nemaline myopathy (NM) is a skeletal muscle disease defined by the presence of sarcomeric disruption and electron dense structures within the sarcomere and/ or nucleus of muscle fibers called rods. These rods are thought to be formed by the unchecked expansion of the α-actinin-rich Z-line. Understanding the mechanism of disruption of the filament systems in muscles that causes nemaline myopathy is of great interest. Candidate molecules that could participate in this mechanism are members of the Four and a half LIM (FHL) domain protein family present in skeletal muscle, FHL1 & FHL3. In particular, FHL3 has been shown to disassemble actin fibers in cell cultures and to inhibit α-actinin bundling of actin. Indeed, Affymetrix array analysis of nemaline patients showed an increase in FHL3 mRNA. We have determined that both FHL1 and FHL3 proteins are upregulated in muscles of our mouse model for nemaline myopathy. In normal muscles, the FHLs localize to the mitochondria, Z- and M-line of the myofiber. In NM muscles, FHL1 and FHL3 no longer co-localize with mitochondria. FHL1 translocates to the nucleus, reminiscent of its localization in immature muscle. In contrast, FHL3 is present in nemaline rods, suggesting a possible role in sarcomeric disruption. Additional features of NM are hypertrophy of specific fibers and moderate, segmental regeneration/repair. The presence of FHL1 in the nuclei may reflect a role in these processes. To address this, localisation of FHL1 was assessed in overloaded, hypertrophied plantaris and regenerating EDL muscles. Nuclear localization of FHL1 was detected in both paradigms. Taken together, these data suggest a role for FHL1 in both hypertrophic and regenerative pathways and for FHL3 in sarcomeric disruption in NM.

1726

**Targeted Ablation of the Mouse Smooth Muscle Gamma-Actin Gene in Mice**

J. C. Szucsik,[1] K. Crawford,[1] J. Lorenz,[2] R. Paul,[2] K. Stringer,[3] E. Brunskill,[4] A. G. Lewis,[5] J. L. Lessard[1]; [1]Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [2]Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, OH, [3]Pathology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, [4]Internal Medicine, The University of Cincinnati College of Medicine, Cincinnati, OH, [5]Pediatric Urology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

The actins are one of the most highly conserved protein families known. In higher vertebrates the actin gene family consists of at least six members, two cytoplasmic isoforms beta- and gamma-actin, as well as four muscle isoforms: cardiac-, enteric-, skeletal-, and vascular-actin. Here, we report the gene targeting of the enteric or smooth muscle gamma-actin (SMGA) gene in mouse embryonic stem cells and the subsequent generation of SMGA null mice. A gene-targeting construct was created to remove the first 60 amino acids of the gene. The construct was electroporated into embryonic stem (ES) cells and approximately 275 colonies were screened. A single ES cell colony with a correctly targeted SMGA allele was identified, expanded, and cells used for blastocyst injections and the generation of chimeric mice. Germline transmission of the targeted SMGA allele was seen in several chimeras. Matings between SMGA heterozygous mice resulted in the predicted Mendelian inheritance pattern of 1:2:1 of wild-type: heterozygous: null offspring. The SMGA null mice have a normal lifespan and are fertile. Matings between null males and null females resulted in normal size litters. The SMGA null mice are hypertensive and demonstrated a significantly higher mean arterial pressure than wild-type controls. Histological examination of smooth muscle containing tissues did not reveal any morphological differences between the heterozygous or null mice compared to wild-type littermates to account for this finding. Our results indicate that a functional SMGA gene is not required for development or survival, similar to the phenotype of the vascular actin null mice, and in sharp contrast to the lethal phenotype seen in cardiac- and skeletal-actin null mice. Like vascular-actin, SMGA is required for proper blood pressure regulation.

1727

**Generation of a Mouse Lacking the C-Terminal of ε-Sarcoglycan using Poly-A Trap Strategy with a Retrovirus Vector**

M. Imamura,[1] T. Sasaoka,[2] Y. Ishida,[3] S. Takeda[1]; [1]Department of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo, Japan, [2]Laboratory of Neurochemistry, National Institute for Basic Biology, Okazaki, Japan, [3]Division of Gene Function in Animals, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Japan

Sarcoglycans (α, β, γ and δ) are transmembrane glycoprotein components of the dystrophin-associated glycoprotein complex, which is critical for the stability of the striated muscle cell membrane. ε-Sarcoglycan (SG) was identified as a homologue of α-SG that is encoded in the causative gene for limb-girdle muscular dystrophy type 2D. However, unlike α-SG, mutations in its gene cause myoclonus-dystonia syndrome but not muscular dystrophy. This indicates the importance of ε-SG for the function in the central nervous system. We found the expression of two ε-SG isoforms in the brain under alternative splicing: one is the conventional type of ε-SG (ε-SG 1) and the other is a brain-specific type (ε-SG2) (Mol. Brain Res. 2004, 125: 1-12). These isoforms are characterized by their different C-terminal sequences and different localization in synaptosomal membranes. Therefore, we hypothesized that these unique structures at the C-terminus are related to individual specific roles at synapses in neuron. To test this, we generated a mouse lacking the C-terminal structures of ε-SGs by poly-A trap strategy. In the bank of gene-trapped mouse ES cells, we found a cell clone having integration of a removable exon trap (RET) retrovirus vector into the *ε-SG* gene (Nucleic Acids Res. 2005, 33: e20) and then generated gene-targeted mice using the ES cell clone. Analysis of genomic DNA of the mice indicated the RET vector integration into intron 11 of

the *ε-SG* gene, resulting in deletion of C-terminal unique sequences of both ε-SG isoforms. Moreover, protein analysis of the mouse brain homogenate also suggested loss of the C-terminal sequences of the isoforms. In conclusion, we have successfully obtained a mouse model for studying the function of ε-SG in the central nervous system.

1728

### *In vitro* and *In vivo* Efficacy of the Cardiac Myosin Activator CK-1827452

R. L. Anderson, S. H. Sueoka, H. M. Rodriquez, K. H. Lee, D. R. Cox, B. P. Morgan, R. Sakowicz, D. J. Morgans, F. Malik, K. A. Elias; Cytokinetics, Inc, South San Francisco, CA

Current inotropic therapies improve contractility by increasing the calcium transient via the adenylyl cyclase pathway, or by delaying cAMP degradation through inhibition of phosphodiesterase (PDE). However, these treatments can be detrimental to patients with heart failure. A preferred approach to improving cardiac contractility may be to directly activate cardiac myosin without changing the calcium transient. Utilizing biochemical assays, we identified compounds (cardiac myosin activators) that directly activate the cardiac myosin ATPase. The cellular mechanism of action, *in vivo* cardiac function in Sprague Dawley (SD) rats, and efficacy in SD rats with defined heart failure to CK-1827452 was then determined. Cellular contractility was quantitated using an edge detection strategy and calcium transient measured using fura-2 loaded adult rat cardiac myocytes. Cellular contractility increased over baseline after exposure to CK-1827452 (0.2 μM) without altering the calcium transient. Combination of CK-1827452 with isoproterenol (β-adrenergic agonist) resulted only in an additive increase in contractility with no further change in the calcium transient demonstrating CK-1827452 is not inhibiting the PDE pathway. *In vivo* contractile function in anesthetized SD rats was quantitated using echocardiography (M-mode) and simultaneous pressure measurements. SD rats were infused with vehicle or CK-1827452 at 0.25 - 2.5 mg/kg/hr. CK-1827452 significantly increased fractional shortening (FS) and ejection fraction (EF) in a dose-dependent manner with no significant change in peripheral blood pressure or heart rate. Rats with defined heart failure induced by left coronary ligation, or sham treated rats had similar and significant increases in FS and EF when treated with CK-1827452. In summary, CK-1827452 increases cardiac contractility without increasing the calcium transient and is efficacious in a rat model of heart failure. These data indicate that CK-1827452 may be a useful therapeutic in the treatment of human heart failure.

1729

### A Multiplexed and Automated Imaging Assay of Cardiac Myocyte Contractility

D. Lenzi, A. Fritsch, G. Alexander, R. Hansen, G. Cong, W. Zink, G. Godinez, A. Pham, J. Trautman, D. Pierce, F. Malik, J. T. Finer; Assay Development, Cytokinetics Inc., South San Francisco, CA

Cardiac myocyte contractility has traditionally been measured using single cell assays. In order to increase throughput and statistical power and facilitate quantitative measurement of the effects of small molecules on cardiomyocyte contractility, we have invented an automated imaging assay that achieves a throughput of more than a thousand cells per experimentalist-day. We took a parallel approach in which multiple cells are measured at once and multiple conditions explored within a computer-controlled experiment. Acutely-dissociated rat ventricular cardiomyocytes are maintained on the stage of an inverted microscope with environmental control in an array of multiple perfusion chambers with integrated electrodes for field stimulation. Imaging is accomplished using a low-magnification objective, and up to 30 contracting cells are imaged per field. Using a motorized stage, multiple fields are imaged per chamber. Custom control software interleaves movie acquisition before and after compound exposure. Movies are taken at high spatial and temporal resolution using a CCD camera and analyzed using custom segmentation and peak-detection algorithms. Distributions of systolic and diastolic lengths are quantified pre and post compound exposure, and dose-response curves computed based on changes in the distributions of contractile behavior. We have successfully used this platform to characterize compounds.

1730

### Hic-5 is a Mediator of Tensional Force, Translocating from Focal Adhesions to Actin Stress Fibers upon Mechanical Stress and Regulating the Contractile Capability of Cells

J. Kim-Kaneyama, M. Shibanuma, K. Nose; Microbiology, Showa University, Tokyo, Japan

Hic-5 is a focal adhesion protein belonging to the paxillin LIM family and shuttling in and out of the nucleus. In the present study, we examined the expression of Hic-5 among mouse tissues by immunohistochemistry and found its exclusive expression in smooth muscle cells in several tissues. This result was consistent with a previous report on adult human tissues and in contrast to the relatively ubiquitous expression of paxillin, the protein most homologous to Hic-5. One factor characterizing smooth muscle cells in vivo is a continuous exposure to mechanical stretch in the organs. To study the involvement of Hic-5 in cellular responses to mechanical stress, we exposed mouse embryo fibroblasts to a uni-axial cyclic stretch and found that Hic-5 was relocalized from focal adhesions to stress fibers through its C-terminal LIM domains during the stress. In sharp contrast, paxillin did not change its focal adhesion-based localization. Among the factors tested including interacting partners of Hic-5, only CRP2, an LIM-only protein expressed in vascular smooth muscle cells, and GIT1 were localized to stress fibers, like Hic-5, during the cyclic stretch. Interestingly, Hic-5 showed a suppressive effect on the contractile capability of cells embedded in three-dimensional collagen gels, and the effect was further augmented by CRP2 co-localized with Hic-5 to fiber structures of those cells. These results suggested that Hic-5 was a mediator of tensional force, translocating directly from focal adhesions to actin stress fibers upon mechanical stress and regulating the contractile capability of cells in the stress fibers.

1731

### Heat Shock-related Protein 20 (hsp20) is a Key Player in Airway Smooth Muscle Relaxation

M. Hinojosa-Kurtzberg, J. Thresher, P. Komalavilas, C. M. Brophy; Bioengineering, Arizona State University, Tempe, AZ

Airway contraction (bronchoconstriction) is a central feature in asthma. Circulating catecholamines and free 5-hydroxytryptamine (5-HT) in the plasma are increased during asthma attacks that correlate with bronchoconstriction and clinical severity. Activation of cyclic nucleotide pathways by nitrovasodilators or $\beta$ -adrenergic agonists leads to phosphorylation of the small heat shock-related protein 20 (HSP20) on Ser 16 mediating vascular smooth muscle relaxation. We have demonstrated earlier that transducible HSP20 phospho- peptides relax vascular smooth muscle from various species after muscarinic and serotonergic agonist-induced contraction. We hypothesized that HSP20 is phosphorylated in response to

increased cyclic nucleotide levels in airway smooth muscle (ASM) mediating relaxation. Bovine airway smooth muscle strips were isolated and treated with contractile agents and vasodilators in a muscle bath followed by biochemical analysis of the phosphorylation of HSP20. Treatment with methacholine ($10^{-8}$-$10^{-5}$M) or 5-HT ($10^{-8}$-$10^{-5}$M) led to a dose dependent contraction of airway SMC. In muscles precontracted with methacholine, isoproterenol (IPT), a β-adrenergic agonist which activates cAMP pathways, induced a dose-dependent relaxation with complete relaxation at $10^{-5}$ M. In ASM precontracted with 5-HT ($10^{-5}$M), sodium nitroprusside, a nitrovasodilator that activates cGMP pathways induced a dose dependent relaxation with complete relaxation at $10^{-}$5 M. There were no increases in the phosphorylation of HSP27 or HSP20 with methacholine or 5-HT induced contractions. However treatment of 5-HT- precontracted airway SMC with IPT was associated with increased HSP20 phosphorylation (72% of total HSP20 protein). Moreover, phosphopeptide analogues of HSP20 led to significant relaxation (63% of maximal contraction) of ASM. These data support a role for HSP20 in mediating airway smooth muscle relaxation and suggest that proteomic approaches may be developed for treatment of asthma.

1732

**Full-length and Peptide Analogs of Phosphorylated HSP20 are Effective Elicitors of a Relaxed Phenotype in Intact Vascular Smooth Muscle and Isolated Cell Systems**

C. R. Flynn,[1] C. Smoke,[1] P. Komalavilas,[1] J. Thresher,[1] M. Guo,[2] R. Penn,[2] C. Brophy[1]; [1]Harrington Department of Bioengineering, Arizona State University, Tempe, AZ, [2]Center for Human Genomics, Wake Forest University Health Science Center, Winston-Salem, NC

We have shown previously that cyclic nucleotide dependent relaxation of vascular smooth muscle is mediated by the small heat-shock-related protein HSP20. This protein is phosphorylated by two different kinases, namely protein kinase A (PKA) and protein kinase G (PKG). In isolated cells phosphorylated (S16) HSP20 (pHSP20) is known to associate with G-actin and other cytoskeletal adapter proteins leading to stellation and a reduction in stress fibers. In intact tissues, pHSP20 inhibits the development of force in response to contractile agonists but does not decrease energy consumption or the phosphorylation of $MLC_{20}$. In this study, transducible HSP20 analogs were generated *ex vivo*, characterized and analyzed for their ability to elicit a relaxed phenotype in both intact tissues and isolated cell systems. cDNAs encoding the catalytic subunit of PKG and HSP20 with an appended amino terminal protein transduction domain (PTD-HSP20) were co-expressed in bacteria and yielded an *in vivo* phosphorylated full-length HSP20 analog as revealed by phospho-specific antibodies and two-dimensional PAGE. A 57% inhibition of a 5-HT-induced contraction of male rat artery was observed after pretreatment with this PTD-pHSP 20. Western blots confirmed the recombinant PTD-pHSP20 was transduced into the vascular strips and that $MLC_{20}$ phosphorylation remained unchanged. Immunofluorescence microscopy of human airway smooth muscle (HASM) cells stably-transfected with a GFP marker and incubated with a peptide mimicking PTD-pHSP20 revealed a time-dependent progression of localization from the cell boundary membrane to the perinuclear region. This localization was commensurate with stellation and a reduction in stress fiber formation. These results support the premise that *ex vivo* synthesized transducible HSP20 analogs are effective in regulating vasomotor tone and do so in a manner downstream of events associated with cyclic nucleotide relaxation.

1733

**Protein Kinase A Inhibition Reverses Forskolin Induced Phosphorylation of Heat Shock-related Protein 20 and Disruption of Stress Fibers in Human Airway Smooth Muscle Cells**

P. Komalavilas,[1] C. R. Flynn,[1] J. Thresher,[1] M. Guo,[2] R. Penn,[2] M. A. Pallero,[3] J. E. Murphy-Ullrich,[3] C. M. Brophy[1]; [1]Harrington Department of Bioengineering, Arizona State University, Tempe, AZ, [2]Center For Human Genomics, Wake Forest University, Winston-Salem, NC, [3]Pathology, University of Alabama at Birmingham, Birmingham, AL

Activation of cyclic nucleotide signaling pathways leads to phosphorylation of the small heat-shock- related protein 20 (HSP20) and actin cytoskeletal changes resulting in the relaxation of vascular smooth muscle. We hypothesized that HSP20 is phosphorylated by protein kinase A (PKA) in response to cAMP elevation in airway smooth muscle cells and leads to disruption of stress fibers. Treatment of human airway smooth muscle (HASM) cells (GFP) with forskolin or isoproterenol that elevate cAMP levels led to increases in HSP20 phosphorylation and loss of stress fibers. HASM cells in which PKA is inhibited (expressing protein kinase inhibitor PKI-GFP), forskolin or isoproterenol failed to increase the phosphorylation of HSP20. There was no disruption of stress fibers in PKA inhibited cells compared to control. Phosphorylation of VASP was also inhibited in cells expressing PKI. Forskolin and isoproterenol decreased phosphorylation of cofilin, the actin depolymerizing protein in control HASM cells, but not in PKA inhibited cells. Forskolin also inhibited focal adhesion formation in control cells but not in PKI-GFP. There was upregulation of HSP20 and protein kinase G in PKA inhibited cells compared to control, however the expression of HSP20 and PKG were lost upon continuous passage of cultures. These results suggest that activation of cAMP pathways leads to phosphorylation of HSP20 and dephosphorylation of cofilin resulting in the disruption of actin stress fibers in HASM and that the effect is mediated by PKA.

## Kinesin I (1734-1748)

1734

**ZBP1 Mediates mRNA Granule Localization Through a Direct Interaction with a Novel Kinesin Light Chain-like Protein in Neurons**

J. B. Dictenberg,[1] K. J. Verhey,[2] R. H. Singer,[3] G. J. Bassell[1]; [1]Neuroscience and Anatomy/Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI, [3]Anatomy/Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Subcellular localization of mRNAs and local protein synthesis are important for process outgrowth and plasticity in neurons. However the molecular details of mRNA-motor interactions remain unknown. This report reveals a direct interaction between the microtubule motor kinesin and an integral component of RNA granules, the zipcode-binding protein 1 (ZBP1) which binds to the 53-nt element (zipcode) within the 3'UTR of beta-actin mRNA and localizes in neurites and growth cones in response to synaptic stimulation. ZBP1 associated with microtubules in a nucleotide-sensitive manner. Immunofluorescence microscopy in hippocampal neurons and immunoprecipitations from brain demonstrated that ZBP1 associated with kinesin heavy (KHC) and light chains (KLC) in dendrites and growth cones but not with other kinesin isoforms. Beta-actin but not vinculin mRNA bound to KHC and KLC in a "zipcode"-dependent manner. Yeast two-hybrid analysis revealed ZBP1 bound to kinesin-I through a novel, phylogenetically conserved KLC-like adapter protein that was enriched in mRNA granules localized to growth cones and

dendrites. Domain mapping showed this protein bound to the cargo-binding domain of KLC through a TPR-TPR interaction and to the N-terminus of ZBP1 containing 2 RRM domains. Importantly, inhibition of kinesin-I subunits and this novel KLC-interacting protein in fixed and living hippocampal neurons (using antisense, dominant negative, and RNAi) disrupted BDNF-stimulated ZBP1 and mRNA transport into dendrites and growth cones, while total poly-A mRNA and vesicular cargoes known to interact with KLC (JIP1) were not affected. These data suggest a model by which ZBP1 functions as a receptor within mRNA granules by binding to the motor directly through this novel KLC-like adapter to localize beta-actin mRNA and possibly many other mRNAs to dendrites in response to neurotrophic signals involved in cytoskeletal remodeling.

1735

**Transport of Mitochondria Requires a Complex of Milton and Miro to Recruit Kinesin Heavy Chain, but does not Require Kinesin Light Chain**

E. E. Glater, L. J. Megeath, T. L. Schwarz; Division of Neuroscience, Children's Hospital and Harvard Medical School, Boston, MA

Mitochondria are distributed within cells in order to match local energy demands. Milton, a *Drosophila* protein, is essential for transport of mitochondria. In homozygous *milton* mutant larvae, mitochondria are absent from axons, but other cargoes, such as synaptic vesicles, are present. We now report that milton acts as an adaptor protein that can recruit the heavy chain of conventional kinesin-1 (KHC) to mitochondria. We have mapped the functional domains of milton: an amino-terminal domain (aa 138-450) is required for binding to KHC; a carboxyl-terminal domain (aa 847-1116) is sufficient for milton's localization to the mitochondria. Biochemical and genetic evidence demonstrate that kinesin recruitment and mitochondrial transport are independent of kinesin light chain (KLC): KLC antagonizes milton's association with KHC in immunocytochemistry and co-immunoprecipitation experiments, KLC is absent from milton-KHC complexes, and mitochondria are present in *klc*$^{-/-}$ photoreceptor axons. The recruitment of KHC to mitochondria is in part determined by the amino-terminus splicing variant of milton. All four forms of milton localize to mitochondria, but only three of the four splice variants recruit KHC in transfected cells. The variant (milton-C) that does not recruit KHC does, however, contain the KHC-association domain, suggesting that its amino-terminus can inhibit the association with KHC. In addition, milton was found to bind to miro, a mitochondrial Rho-like GTPase, in a yeast two hybrid screen of the *Drosophila* proteome (Giot *et al.,* 2003 Science 302: 1727-36). We have confirmed this interaction with co-immunoprecipitation experiments from transfected cells and have shown that miro governs the recruitment of milton and hence KHC to mitochondria. Milton and miro thus form an essential protein complex that links KHC to mitochondria for KLC independent, anterograde transport of mitochondria.

1736

**Single Molecule Analysis of Kinesin 1 Activation through Cargo Binding**

C. Huang, B. J. Schnapp; Cell & Developmental Biology, Oregon Health & Science University, Portland, OR

Kinesin 1 is present largely as soluble tetramer of two heavy chains and two light chains that is self-inhibited with respect to ATPase activity and microtubule binding. Self-inhibition depends on folding of the kinesin heavy chains, which allows for an intra-molecular interaction between its C-terminal residues and the N-terminal motor/ neck regions. The kinesin light chains are also needed for full inhibition in vivo. Much less is understood about how Kinesin 1 becomes activated for cargo transport. To address whether activation occurs directly through specific cargo binding, we have examined how the motility of single recombinant GFP-tagged Kinesin 1 tetramer is affected by the binding of defined cargo-linker molecules to its two known cargo binding sites. JIP and SNAP25 recognize and bind to the light chain TPRs and the heavy chain cargo binding domain, respectively. Addition of a JIP-C-terminal peptide increases the frequency of Kinesin 1 motility events without significantly affecting their velocity (~8µm/min). Addition of SNAP25 principally affects velocity. In the presence of SNAP25, the distribution of the velocity of motility events appears to be bimodal with a minor peak at ~20µm/min in addition to the major 8µm/min peak. These findings suggest that each cargo can relieve the self-inhibition of kinesin through different mechanisms. This is parallel to previous reports that light chain inhibits the binding of tetramer to the microtubule and that the tail domain of heavy chain represses motor activity. We conclude that cargo binding itself can activate conventional kinesin activity and that the coordination of light chain and heavy chain cargo binding may be an importlant way of regulating kinesin activities and hence intracellular transport in vivo.

1737

**Fluorescence Resonance Energy Transfer (FRET) Stoichiometry Reveals Kinesin-1 Activation Mechanism in Live Cells**

D. Cai,[1] A. Hoppe,[2] J. Swanson,[2] K. Verhey[1]; [1]Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, [2]Microbiology and Immunology, University of Michigan, Ann Arbor, MI

In the absence of cargo, kinesin-1 is inactive for microtubule-based motility. Previous work has suggested that kinesin-1 is able to adopt distinct conformations *in vitro*. Based on these observations, it has been hypothesized that the inhibited kinein-1 is folded whereas the active kinesin-1 is extended. To reveal kinesin-1 conformational changes upon inhibition-activation in living cells, we used FRET stoichiometry to study Fluorescent Protein-labeled kinesin-1 molecules. When expressed in COS cells, kinesin-1 is not active for microtubule binding and is in a tightly folded conformation. In the absence of kinesin light chain (KLC), the kinesin heavy chain (KHC) dimer is no longer tightly folded and is active for microtubule binding. The two motor domains of the active KHC are closer together, presumably in the right spatial arrangement to bind to adjacent ÃŽÂ²-tubulin subunits of a microtubule protofilament. This implies that KLC polypeptides force the two KHC motor domains apart in the folded inactive motor. Truncation of the KHC tail domain resulted in a molecule that, although folded with the KHC motor domains pushed apart, was active for microtubule binding. Thus, the KHC tail domain contributes to auto-inhibition by blocking the microtubule binding site of the KHC motor domain. These results support the hypothesis that kinesin-1 undergoes a global conformational change *in vivo*, from folded and inhibited to extended and activated. Our results also suggest an important local conformational change between the two KHC motor domains upon activation. We propose that release of both KLC polypeptides and the KHC tail domains from the KHC motor domains is required for activation of motor activity *in vivo.*

1738

**Fasciculation and Elongation Protein ζ-1 (FEZ1) Plays a Role in the Activation of Kinesin-1**

K. Verhey, G. Jih, L. Blasius; Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

In the absence of cargo, kinesin-1 is auto-inhibited due to a direct interaction of the kinesin heavy chain (KHC) tail domain with the KHC motor domain. How kinesin-1 is activated upon cargo binding is unknown. We tested the possibility that cargo binding activates the motor for microtubule-based motility. Binding of kinesin-1 to the cargo molecule JNK Interacting Protein 1 (JIP1) was not sufficient to activate kinesin-1 for microtubule binding. Given these results. we hypothesized that additional proteins may be required for kinesin-1 activation. We identified fasciculation and elongation protein ζ-1 (FEZ1), the mammalian orthologue of *Caenorhabditis elegans* UNC-76, as a binding partner of KHC. FEZ1 binds to the inhibitory coiled region of the KHC tail domain, adjacent to the IAK residues required for auto-inhibition, suggesting that FEZ1 plays a role in regulating kinesin-1 activity. Consistent with this, binding of both JIP1 and FEZ1 to kinesin-1 was sufficient to activate the motor for microtubule binding. These results suggest that FEZ1 plays a role in activation of kinesin-1 upon cargo binding.

1739

**Microtubule Acetylation Dictates Kinesin-1 Binding to Microtubules and Transport in Neuronal Cells**

N. A. Reed,[1] G. W. Jih,[1] J. Gaertig,[2] K. J. Verhey[1]; [1]Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, [2]Department of Cellular Biology, University of Georgia, Athens, GA

Motor proteins use the energy of ATP hydrolysis to ferry cellular cargoes along microtubules and actin filaments. In neurons, the delivery of membrane and cytoplasmic cargoes must be coordinated with the decision of the growth cone to advance, pause or retract. We show using Fluorescent Recovery After Photobleaching (FRAP) that, in differentiating neuronal cells, kinesin-1 (formerly conventional kinesin or Kif5) specifically delivers the cargo molecule JNK-interacting protein 1 (JIP1) to growing neurites but not to retracting neurites. The mechanism for this distinction appears to be a specific post-translational modification (PTM) of microtubule filaments. Kinesin-1 binds *in vitro* selectively to microtubules marked by acetylation of α-tubulin at residue lysine-40 and JIP1 transport *in vivo* is increased by pharmacological treatments that increase microtubule acetylation. These data suggest that microtubule PTMs play an important role in directing protein trafficking to specific subcellular locales.

1740

**Kinesin-1 Heavy Chain Preferentially Binds to and Moves Along Detyrosinated Microtubules in Living Cells**

S. Dunn,[1] H. Musa,[1] E. E. Morrison,[2] M. Peckham[1]; [1]Biomedical Sciences, University of Leeds, Leeds, United Kingdom, [2]CRUK Clinical Research Centre, St James University Hospital, Leeds, United Kingdom

Kinesin-1 (conventional kinesin) is a microtubule-dependent motor involved in the transport of diverse cargoes. We investigated the dynamic properties of kinesin-1 using a GFP-tagged kinesin-1 heavy chain (GFP-Kif5c) *in vivo*. By live cell imaging, we show that GFP-Kif5c is a processive plus-end directed microtubule motor that moves at speeds of up to 1µm $s^{-1}$ *in vivo*, similar to speeds reported *in vitro*. Significantly, GFP-Kif5c only labeled a subset of microtubules that we think are likely to be detyrosinated (Glu-tubes) for the following reasons. First, the appearance and behaviour of these microtubules in live cells suggested that they are stable microtubules. Second, GFP-Kif5c accumulates on and moves along drug-resistant microtubules following nocodazole-induced microtubule depolymerisation in transfected cells. Finally, co-immunostaing suggests that they are composed of detyrosinated tubulin. The function of detyrosinated microtubules inside cells has remained unclear since their discovery over 20 years ago. Our results suggest that the cells may use this biochemically distinct subset of microtubules to direct kinesin-1 dependent trafficking, which in turn could be regulated by signaling pathways.

1741

**Kinesin-1 Transports Phosphorylated and Non-phosphorylated Amyloid-*β* Precursor Protein (APP) by Distinct Pathways**

Z. Muresan, V. Muresan; Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

Kinesin-1 carries the transmembrane protein, APP (the precursor of the amyloid-β peptide that forms the senile plaques in Alzheimer's disease) to the axon terminal. A fraction of the total APP is transported after it is phosphorylated at Thr668 by c-Jun NH2-terminal kinase (JNK), a process that occurs in the cell body and is facilitated by JNK-interacting protein-3 (JIP-3). Using the central nervous system-derived, neuronal cell line, CAD, we show that phosphorylated APP (pAPP), but not non-phosphorylated APP, recruits kinesin-1 via JIP-1, a JNK scaffolding protein unrelated to JIP-3. Thus, pAPP serves as anchoring site and carrier for a large signaling complex that includes JIP-1, JIP-3, activated JNK, and kinesin-1, which becomes localized to the growth cone. By contrast, we find that Fe65, another APP binding protein that binds kinesin-1, forms a complex in the cell body, and is co-transported into neurites strictly with the fraction of APP that is not phosphorylated at Thr668. The two complexes are transported to different sites at the neurite terminal. Consistent with these results, down-regulation of JIP-1 by siRNA specifically impairs transport of pAPP, with no effect on the trafficking of non-phosphorylated APP. In biochemical experiments, we find that JIP-1 preferentially interacts with pAPP, compared to non-phosphorylated APP. By contrast, Fe65 binds with higher affinity to non-phosphorylated APP, compared to pAPP. We show that, in the cellular context, Fe65 prevents JIP-1 from binding to non-phosphorylated APP, but not to pAPP, a result that explains mechanistically the recruitment of kinesin-1 via JIP-1 to pAPP and via Fe65 to non-phosphorylated APP. Thus, APP transport into axons occurs via two separate populations of vesicles that differ in composition, and whose transport is differentially regulated by phosphorylation of APP.

1742

**Regulation of *drosophila* KLP10A Localization and Function by Phosphorylation**

V. Mennella, A. Asenjo, H. Sosa, D. Sharp; Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY

Microtubule dynamics must be precisely regulated both in time and in space in order for cells to perform vital functions during the course of their lifespan. A subfamily of kinesins, termed Kinesin 13s (Kin I), have been found to be important effectors of microtubule depolymerization. Recent studies focusing on the cellular functions and dynamics of a Drosophila kinesin 13 family, KLP10A, have revealed complex regulation of this enzyme. Indeed, the targeting and activation of KLP10A appears to vary dramatically through the course of the cell cycle and among distinct subsets of cytoplasmic microtubules. In an effort to understand the underlying mechanism of this regulation, we are mapping the sites of phosphorylation of KLP10A occuring *in vivo*. Of particular interest, ESI mass spectrometry has revealed phosphorylation of a residue that is conserved throughout the entire Kinesin superfamily, suggesting that it may act as a major regulatory switch for several motors with different

functions. In addition, we have generated different cell lines exogenously expressing EGFP-KLP10A which bear mutations in this residue to mimic the phosphorylated or non-phosphorylated states. We are currently characterizing the biological activity of these mutants in an endogenous KLP10A null background generated by RNAi. The biochemical characteristics of KLP10A mutants are also being assessed in vitro.

1743

**The Depolymerizing Kinesin MCAK Undergoes 1D Diffusion to Target the Microtubule Ends**

G. J. Brouhard, J. H. Helenius, Y. Kalaidzidis, S. Diez, J. Howard; Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany

MCAK, a member of the kinesin-13 family of motor proteins, depolymerizes microtubules from both ends and regulates microtubule dynamics in both mitosis and interphase. The depolymerization reaction is fast, reaching a plateau rate of 4 μm/min, equivalent to the removal of 60 tubulin subunits/s per end; and the association rate to the ends is remarkably high, at about 750 x $10^6$ $M^{-1}s^{-1}$. This high rate of targeting to the microtubule ends is not understood, especially considering that MCAK shows no directed motility on the microtubule lattice. To address the question of how MCAK targets the microtubule ends so quickly, we have developed an assay for observing the activity of single MCAK motors using total-internal-reflection-fluorescence microscopy. In this assay, baculovirus-expressed MCAK-EGFP shows an ATP-dependent depolymerization of GMP-CPP microtubules from both ends. Rather than walking along the microtubule lattice, MCAK undergoes a random motion; the diffusion constant of this motion is 0.4 $\mu m^2/s$, and the half-life of lattice binding is 0.6 s, measured for >1000 single molecules. The 1D diffusion of MCAK creates a "reduction in dimensionality" that allows rapid targeting to microtubule ends, with a predicted flux of MCAK to each end on the order of 10 $s^{-1}$ at 10 nM MCAK, or 1000 x $10^6$ $M^{-1}s^{-1}$. This is sufficiently high to account for the association rate to the ends and for the rapid depolymerization of the microtubule.

1744

**MCAK Domains Essential for Microtubule Depolymerization Activity During Spindle Assembly**

S. C. Ems-McClung,[1] K. M. Hertzer,[1] M. W. Miller,[2] C. E. Walczak[1]; [1]Medical Sciences Program, Indiana University, Bloomington, IN, [2]Biological Sciences, Wright State University, Dayton, OH

MCAK, a Kinesin-13, depolymerizes microtubules rather than translocating along them. Similar to other kinesins, MCAK activity is regulated by domains outside of the catalytic domain. While several studies have examined the putative function of these regulatory regions, it remains unclear which domains are most critical to control microtubule depolymerization activity and how this correlates with MCAK activity during spindle assembly. We expressed and purified eight different MCAK deletion and/or catalytic domain point mutation constructs from insect cells and analyzed their depolymerization activity using a dose-response assay. We found that the MCAK neck domain and C-terminal domain are important for robust *in vitro* microtubule depolymerization, whereas the far N-terminus is not necessary. When the far N-terminus and C-terminal domain deletions are combined into our minimal domain construct, depolymerization activity is further compromised. This is in contrast to cellular transfection assays, in which the far N-terminus is important, the C-terminal domain is not important, and minimal domain functions as well as full-length MCAK. Interestingly, our catalytic domain point mutant proteins showed differential depolymerization activities. To ask how *in vitro* depolymerization activity correlates with the MCAK function during spindle assembly, we immunodepleted MCAK from egg extracts and rescued the depletion with each MCAK derivative. Preliminary results suggest that the ability of an MCAK mutant to rescue physiological microtubule aster formation does not necessarily correlate with *in vitro* depolymerization. The MCAK minimal domain protein has a high affinity for tubulin dimer and may not dissociate freely from tubulin during the catalytic cycle, suggesting that domains outside of the catalytic domain contribute to efficient tubulin release. Additional biochemical analyses on the various constructs are underway to determine where in the catalytic cycle these proteins are acting differentially and how this correlates with physiological spindle formation.

1745

**Studies of Single Molecules of the Mitotic Kinesin Eg5**

B. H. Kwok,[1] L. C. Kapitein,[2] J. H. Kim,[1] C. F. Schmidt,[2] E. J. G. Peterman,[2] T. M. Kapoor[1]; [1]Cell Biology & Chemistry, Rockefeller University, New York, NY, [2]Department of Physics and Astronomy, Vrije Universiteit, Amsterdam, The Netherlands

During cell division, a bipolar mitotic spindle is required to equally segregate the genetic material into two daughter cells. Eg5 (kinesin-5), an evolutionarily conserved member of the BimC kinesin family, plays a key role in the assembly of bipolar spindles in eukaryotes. Recently, in controlled *in vitro* experiments, we have shown that full length homotetrameric recombinant Eg5 can crosslink two microtubules and walk on each filament to slide microtubules apart. Based on these studies we predicted that Eg5 is likely to be a doubly processive motor, walking many steps on each microtubule it crosslinks. To test this hypothesis and to directly observe the movements of single Eg5 molecules on microtubules, we have generated and characterized a Eg5-GFP fusion protein. We find this protein to be tetrameric in solution and show that it can functionally substitute for endogenous Eg5 in spindle assembly assays in *Xenopus* egg extracts. Fluorescence microscopy-based analyses of single Eg5 molecules reveal that Eg5 moves processively along microtubules immobilized on a surface. The average velocity of individual Eg5-GFP tetramers was ~2 microns/min and average runlength ~2.5 μm. These results provide essential input to build quantitative models for how individual molecular motor molecules contribute to mitotic spindle assembly and function.

1746

**The Bipolar Mitotic Kinesin Eg5 Moves on Both Microtubules That it Crosslinks**

L. C. Kapitein,[1] E. J. Peterman,[1] B. H. Kwok,[2] J. H. Kim,[2] T. M. Kapoor,[2] C. F. Schmidt[2]; [1]Department of Physics and Astronomy, Vrije Universiteit, Amsterdam, The Netherlands, [2]Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY

During cell division, mitotic spindles are assembled by microtubule-based motor proteins. The bipolar organization of spindles is essential for proper segregation of chromosomes and requires plus-end directed homotetrameric motor proteins of the widely conserved kinesin-5 (BimC) family. Models for bipolar spindle formation include the "push-pull mitotic muscle" model in which kinesin-5 and opposing motor proteins act between overlapping microtubules. The precise roles, however, of kinesin-5 during this process are unknown. Using controlled *in vitro* assays, we studied how the vertebrate kinesin-5 Eg5 operates between microtubules. We found that this motor drives sliding of crosslinked microtubules

dependent on their relative orientation. This is possible because Eg5 has the remarkable capability of simultaneously moving at ~20 nm/s towards the plus-ends of each of the two microtubules it crosslinks. For anti-parallel microtubules, this resulted in relative sliding at ~40 nm/s, comparable to spindle pole separation rates *in vivo*. Furthermore, we found that Eg5 can tether microtubule plus-ends, suggesting an additional microtubule-binding mode for Eg5. Currently, we are using single-molecule imaging of Eg5-GFP to test whether single processive motors can establish the observed sliding and end-tethering. These results demonstrate how kinesin-5's are likely to function in mitosis, pushing interpolar microtubules apart, as well as recruiting microtubules into bundles that are subsequently polarized by relative sliding.

1747

**Chemical and Genetic Disruption of *Candida albicans* Kip1p: A Unique Bipolar Kinesin**

D. M. Roof, P. R. Chua, A. L. Tomasi, B. P. Morgan, D. Clarke, D. Morgans, M. Hamilton, R. Sakowicz, D. Pierce, T. Stephens, Y. Lee, M. E. Maxon; Cytokinetics, Inc., South San Francisco, CA

The genome of the pathogenic yeast *Candida albicans* encodes a single member of the bipolar (Kinesin-5) kinesin family, Kip1p. In order to screen for inhibitors of Kip1, we cloned and expressed the *C. albicans* Kip1 motor domain in *E. coli* and obtained a protein with microtubule-stimulated ATPase activity. Screening of a small molecule library and subsequent chemical modifications produced an aminobenzothiazole (ABT) compound which inhibited the Kip1 ATPase activity in an ATP-competitive fashion. This inhibition results in "rigor-like", tight association of Kip1 and microtubules *in vitro*. Upon treatment of *C. albicans* cells with the ABT compound, cell division was blocked and a unique, aberrant spindle morphology was elicited in which multiple spindle poles accumulate in close proximity to each other. Typical of bipolar kinesins in other organisms, we found that *C. albicans* Kip1 localizes to the mitotic spindle. Inducible excision of the Kip1 gene resulted in the transient appearance of a phenotype similar to that induced by the ABT compound. Surprisingly, deletion mutants were able to recover and were viable. The similarity of the effects of genetic and chemical disruption of Kip1p function support the conclusion that the ABT compound has an "on-target" effect on *C. albicans* Kip1 *in vivo*, making it a first in class fungal spindle inhibitor targeted to a mitotic kinesin. In addition, our results suggest that the non-essential nature and implementation of the bipolar motor in *C. albicans* differs from that seen in other organisms.

1748

**Differential Targeting of Related C-terminal Kinesins**

Y. Zhang, K. L. Thompson, R. Wang, A. O. Sperry; Anatomy and Cell Biology, East Carolina Universiy, Greenville, NC

Formation of the spindle pole requires the combined function of numerous structural and motor proteins, including C-terminal kinesins. We have identified a small group of highly related C-terminal kinesin motors in the mouse: KIFC1, KIFC5A, KIFC5B, and KIFC5C. The latter three isoforms are very similar to KIFC1 except for two unique sequence inserts in their tail domain, termed insert 1 and insert 2. Insert 1 of KIFC5A is necessary and sufficient to target this motor to the nucleus and enable it to bind microtubules. We wanted to determine whether the other two isoforms, 5B and 5C, have similar targeting and functional properties as compared to 5A. We propose that the unique sequences present in the B and C isoform might direct these motors to different intracellular locations where they perform distinct functions. Proteins were localized within the cell using isoform specific antibodies and GFP fusion constructs. In order to determine the role of the KIFC5A and 5B isoforms in spindle formation an in vitro microtubule aster assay was used. Our results show that the small changes in tail amino acid sequence targeted the isoforms to different locations within the cell. Also, KIFC5A, and possibly 5C, were found to be important in the formation of asters, while antibodies to KIFC5B had no effect on aster formation in vitro. Taken together, this data supports our hypothesis that minor changes in the tail domain sequences of these motors is sufficient to cause distinct cellular localization and function.

## Microtubule Dynamics & Assembly II (1749-1765)

1749

**Bisphenol A Targets the Microtubule Cytoskeleton in Yeast and Mammalian Cells**

C. Wong, A. C. Ballew, L. Cai, T. Stearns; Biological Sciences, Stanford University, Stanford University, CA

Bisphenol A (BPA) is a synthetic estrogen-related compound that polymerizes into hard, clear polycarbonate plastic that is used widely in consumer products. The BPA polymer is unstable and releases BPA monomers into solutions. BPA and related compounds have been shown to cause meiotic defects in mice, and are cytotoxic to mammalian cells. The mechanism of BPA cytotoxicity is unknown, however, some evidence suggests that BPA might affect the microtubule cytoskeleton. To identify cellular targets of BPA, we performed an induced haploinsufficiency screen in *Saccharomyces cerevisiae*. The heterozygous diploid deletion collection was grown as a pool with or without BPA, and the difference in growth rates between strains was determined using deletion barcode microarrays. The most severely growth-compromised strain was the strain heterozygous for a deletion of the yeast β tubulin, *TUB2*, suggesting that tubulin may be the main target of BPA. Consistent with this hypothesis, BPA also caused an increase in mitotic index and defects in spindle formation in wild-type yeast cells. We also tested the effect of BPA on the microtubule cytoskeleton in human diploid fibroblasts. BPA caused bundling of microtubule filaments and formation of unique ring structures. In an *in vivo* microtubule regrowth assay, BPA inhibited microtubule nucleation at the centrosome, yet enhanced non-centrosomal microtubule growth in the cytoplasm. In an *in vitro* microtubule polymerization assay, BPA caused bundling and branching of microtubules. These results suggest that BPA exerts its cytotoxic effect by altering the structure and dynamic nature of microtubule filaments.

1750

**Resistance to Taxol or to Colcemid Associated with Different Amino Acid Substitutions at P220 of β1-Tubulin**

S. Yin, F. Cabral; Dept. of Integrative Biology and Pharmacology, University of Texas Houston Health Science Center, Houston, TX

Mutations affecting L215 and L217 in the H6/H7 loop of β1-tubulin occur at high frequency in Taxol resistant, but not colcemid resistant cells. To determine whether mutations at other residues in this loop are able to confer drug resistance, saturation mutagenesis of the highly conserved P220 in the loop was carried out. Transfection of a pool of mutagenized β1-tubulin cDNAs with randomized nucleotides at codon 220, followed by selection of the cells in Taxol, resulted in the isolation of cell lines carrying P220L and P220V substitutions. Selection of the transfected cells in colcemid, on the other hand, resulted in the isolation of cells carrying P220C, P220S and P220T. By the use of site-directed mutagenesis and

retransfection, P220L and P220V were confirmed to confer Taxol resistance, while P220C, P220S and P220T were shown to confer colcemid resistance. Consistent with a mechanism of resistance based on changes in microtubule stability, the first set of mutations caused destabilization of microtubules, but the second set caused microtubule stabilization. The results show for the first time that changes in a single amino acid in the H6/H7 loop of β1-tubulin can confer resistance to drugs with opposing actions on microtubule assembly and with distinct binding sites.

1751

**Assembly of β-Tubulin with a 30 Amino Acid Insertion**

H. Yang, F. R. Cabral; Integrative Biology and Pharmacology, University of Texas-Houston Health Science Center, Houston, TX

The N-loop of β-tubulin forms part of the lateral surface of microtubules and protrudes into the microtubule lumen. Prior studies have shown that single mutations in this region can affect microtubule assembly and thus alter the sensitivity of cells to antimitotic drugs. We now report that a 30-amino-acid insertion after residue 63 in the N-loop of class IVa β-tubulin produces a stable protein that is able to incorporate into microtubules. A plasmid containing the mutant βIVa-tubulin cDNA under the control of a tetracycline-regulated promoter was transfected into Chinese hamster ovary cells and stable cell lines with different expression levels were selected. Using immunofluorescence to visualize the microtubule network and biochemical fractionation to separate microtubule polymers from free tubulin dimers, we discovered that the mutant β-tubulin could assemble into microtubules but only at a greatly reduced efficiency. The endogenous β-tubulin assembly was not affected. Cells could tolerate a small amount of the mutant β-tubulin but high expression led to severe growth defects. The results indicate that the mutant β-tubulin folds normally and is able to form heterodimers with α-tubulin. However, mutant heterodimers act as a sink for α-tubulin, thereby lowering the effective concentration of assembly-competent tubulin and the microtubule content of the cell. These studies have identified a region in β-tubulin that can accommodate large insertions, thereby expanding the options for creating assembly-competent tubulin reporter molecules and affinity probes.

1752

**The Protein Kinase MARK/Par-1 is Regulated by the Interaction with the p21-activated Kinase PAK5**

B. Griesshaber, D. Matenia, X. Y. Li, A. Thiessen, J. Jiao, C. Johne, T. Timm, E. Mandelkow, E. M. Mandelkow; Structural Molecular Biology, Max-Planck-Institute, Hamburg, Germany

Protein kinases of MARK/Par-1 family are involved in the development of embryonic polarity, and in neurons they phosphorylate the microtubule-associated protein tau, causing its detachment from microtubules. The target sites of MARK kinases on tau protein occur early in Alzheimer neurodegeneration which prompted us to search for regulatory factors of MARK. A yeast two hybrid screen with the MARK2 isoform yielded several interaction partners, including variants of the scaffolding protein 14-3-3, and the kinase PAK5, a member of the p21-activated kinases which is prominent in brain tissue. Both kinases contain multiple domains, including the typical kinase catalytic domain. A refined two-hybrid search for domain interactions showed that the interaction resides in the two catalytic domains, which are near the N-terminus in the case of MARK2 and near the C-terminus in the case of PAK5. PAK family kinases are activated by small G-proteins (e.g. Rac, Cdc42) and typically regulate the actin cytoskeleton, whereas MARK kinases affect the microtubule network. The interaction of the two kinase domains was confirmed biochemically and by immunoprecipitation from HEK 293 and Sf9 cells. Unexpectedly, the binding of PAK5 strongly inhibited the kinase activity of MARK, but not vice versa. The inhibition of MARK2 was mediated by the binding of PAK5(cat) as such, but not by phosphorylation. This interaction therefore constitutes a possible link between cytoskeletal regulatory pathways affecting the actin and microtubule networks (see Matenia et al., accompanying abstract). - Supported by DFG.

1753

**Ying-Yang of Microfilament and Microtubule Dynamics Regulated by the Interference between PAK and MARK Protein Kinases**

D. Matenia, B. Griesshaber, X. Y. Li, A. Thiessen, J. Jiao, C. Johne, E. M. Mandelkow; Structural Molecular Biology, Max-Planck-Institute, Hamburg, Germany

The dynamics of the microfilament and microtubule cytoskeletons are regulated by multiple pathways including phosphorylation by protein kinases. The PAK family of kinases is known to induce actin-dependent processes (e.g. filopodia, lamellipodia) after activation by small G-proteins of the Rho family. The MARK family of kinases (alias Par-1) plays a role in the generation of cell polarity and in microtubule dynamics (e.g. in neurite outgrowth or growth cone advance). We have recently observed that the brain-specific variant PAK5 binds to MARK family kinases, notably MARK2, and thereby inhibits the kinase activity of MARK2 (see accompanying poster by Griesshaber et al.). Here we report on the cellular effects of this interaction. In transfected CHO cells both kinases show a vesicular distribution with a high degree of colocalization on endosomes containing the adaptor proteins AP1 or 2. Fractionation of vesicle proteins from Sf9 cell expressing MARK2 and PAK5 on an iodixanol gradient revealed colocalization of MARK2 and PAK5 with g-adaptin and b1/b2-adaptin. MARK2 transfected alone destabilizes microtubules since it phosphorylates microtubule-associated proteins, and concomitantly the actin stress fibers are stabilized. By contrast, PAK5 keeps microtubules stable because it downregulates the activity of MARK2, at the same time it destabilizes the F-actin network so that stress fibers and focal adhesions disappear and cells develop filopodia. The results point to an inverse relationship between actin- and microtubule-related signalling by the PAK5 and MARK2 pathways that affect both cytoskeletal networks. - Supported by DFG.

1754

**Microtubule Depolymerization Activity of a Kip3 Kinesin: Results from a Novel High-throughput Microtubule Depolymerization Assay**

R. Baliga, D. Guo, P. Chua, T. Stephens, M. Yu, D. W. Pierce, R. Sakowicz, M. Maxon, J. T. Finer; Assay Development, Cytokinetics, South San Francisco, CA

Kip3 type kinesins are thought to be involved in depolymerization of microtubules (MTs). Consistent with this function, we have discovered that the Kip3 kinesin from *Candida albicans* (CaKip3) is essential, but only at elevated temperatures. Further, in an effort to identify novel inhibitors of fungal kinesins, we cloned and expressed the motor domain of CaKip3 in a bacterial expression system. Using a novel kinetic fluorescence polarization (KFP) assay that enables detection of relatively weak MT depolymerization activity, we found that CaKip3 is able to depolymerize Paclitaxel-stabilized MTs. We miniaturized and automated the KFP assay and screened a ~70000 compound small molecule library for inhibitors of this CaKip3 activity. We identified several modestly potent compounds that stabilize MTs specifically in the presence of CaKip3. In parallel,

using a conventional absorbance-based ATPase screen, we identified inhibitors of the CaKip3 ATPase activity. Interestingly, there is little if any overlap between the two sets of compounds, indicating a possible separation of the ATPase and MT depolymerization activities of this motor. Since CaKip3 is essential in *Candida albicans*, the inhibitors we have identified could provide useful starting points for antifungal drug discovery.

1755

**Two Regulatory Phosphoswitches Control MCAK Function**

X. Zhang,[1] W. Lan,[2] T. Stukenberg,[2] C. E. Walczak[3]; [1]Biology, Indiana University, Bloomington, IN, [2]University of Virginia, Charlottesville, VA, [3]Medical Science, Indiana University, Bloomington, IN

MCAK is a microtubule (MT) depolymerizing enzyme that plays important roles in chromosome alignment and in correcting improper kinetochore-MT attachments. Its activity and localization are regulated by Aurora B kinase. *Xenopus* MCAK is phosphorylated by Aurora B at S196 in the neck region, which results in inhibition of its MT depolymerization activity. We now show that Aurora B phosphorylates T95 in the centromere-targeting domain of MCAK, which regulates its localization to chromosomes. While wild-type GST-NT fusion proteins of MCAK targeted to centromeres, the GST-NT (T95A) mutant assembled large aggregates along chromosomes. These “chromosome nuggets” do not alter the localization of the other kinetochore proteins, such as Ndc80, p150 or CENP E. Interestingly, decreasing the concentration of GST-NT (T95A) added to extracts by 100 fold abolished the formation of these “chromosome nuggets” and instead allowed targeting to centromeres. As a complementary approach, we depleted endogenous MCAK from the extract and added back either full length MCAK or an MCAK-T95A mutant. MCAK-T95A targeted robustly to centromeres when only 10% of the endogenous level of MCAK was added, whereas wt MCAK needed to be added at near endogenous level to target. These results suggest that T95A mutants have a high affinity for the centromere region. We also found that endogenous MCAK associates with chromosome arms. This chromosome arm staining may be mediated by phosphorylation at T95 as a phospho-T95 antibody strongly stains chromosome arms in both extracts and cells, a result confirmed by biochemical analysis of chromatin-coated DNA beads. Our work provides an important example of how a protein can be differentially regulated by multiple site phosphorylation and how these different sites can independently change a protein’s activity.

1756

**Modeling Microtubule Dynamic Instability**

I. V. Gregoretti,[1] A. M. Willis,[2] M. S. Alber,[2] H. V. Goodson[1]; [1]Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, [2]Department of Mathematics, University of Notre Dame, Notre Dame, IN

Microtubules are dynamic tubular polymers that provide cells with a scaffold for internal organization, vesicle trafficking, and cell division. Key to microtubule function is dynamic instability --stochastic switching between phases of individual polymer growth and shortening, observed both in vivo and in vitro. Although dynamic instability has been studied by many approaches, gaining a predictive understanding of a system of dynamic microtubules requires mathematical modeling. Deterministic modeling efforts have had limited utility because they describe the behavior of the population, not of individual filaments. Existing Monte Carlo models simulate only a single microtubule in isolation or lack the ability to explain how characteristics such as transition frequencies and critical concentration arise as a function of simple parameters. In particular, most models ignore competition between microtubules for tubulin subunits. To address these issues, we have developed a Monte Carlo computer model featuring the essential aspects of this process, including competition between filaments and the existence of system boundaries (a cell edge). Transition frequencies evolve from the simulation rather than being set. Our results show that competition between microtubules for tubulin subunits is key to understanding the dynamic behavior of both individual microtubules and the population. In particular, we find that the processive growth of microtubules observed in vivo is an expected outcome of the increase in free tubulin concentration caused by catastrophe induction at the cell edge --regulatory gradients may influence processive growth but are not required for it. Similarly, the experimental observation that mutations in microtubule nucleation machinery alter microtubule dynamics can potentially be explained by changes in the number of competing microtubules. These simulations should enhance understanding of the emergent properties of systems of dynamic microtubules and provide a foundation for better understanding of the activities of microtubule binding proteins.

1757

**Force Production by Disassembling Microtubules**

K. Grishchuk,[1,2] M. I. Molodtsov,[3] F. I. Ataullakhanov,[3,4] R. McIntosh[1]; [1]MCD Biology, University of Colorado, Boulder, CO, [2]Inst. Gen. Path. & Pathophysiol., Moscow, Russian Federation, [3]National Res. Centre for Haematology, Moscow, Russian Federation, [4]Physics, Moscow State Univ., Moscow, Russian Federation

Microtubules (MTs) are important components of the eukaryotic cytoskeleton; they contribute to cell shape and movement and to the motions of organelles, including mitotic chromosomes. MTs bind motor enzymes that drive many such movements, but MT dynamics can also contribute. It has previously been proposed that MTs could generate force through their unusual mechano-chemistry. These polymers assemble from tubulin dimers that bind GTP and hydrolyse it after polymerisation. The GDP-dimer conformation is bent relative to its GTP-counterpart, but this bend is constrained by MT geometry, so some energy from GTP-hydrolysis is stored within the lattice. During depolymerization, the accumulated stress forces the strands of GDP-dimers, or “protofilaments” (PFs), to arch out in a “ram’s horn” configuration. Thus, each MT is a reservoir of chemical energy that can in principle be harnessed to do mechanical work. Two aspects of this mechanism have gone uncharacterised: the power-stroke that is probably generated by curving PFs and the coupling devices that might convert this energy into motility. Here we conjugated glass microbeads to tubulin polymers via strong, inert linkages, like biotin-avidin. Depolymerizing MTs exerted a brief tug on such beads, as measured with laser tweezers. These interactions have been analysed with a molecular-mechanical model for MT structure and force production. We show that a single depolymerizing MT can generate ~10-times the force developed by a motor enzyme, so this mechanism might be the primary driving force for chromosome motion. Even the simpler coupler used here slows MT disassembly; physiological couplers may therefore modulate MT dynamics in vivo.

1758

**Ethanol-induced Impairment of Hepatic Microtubule and Membrane Dynamics May Be Mediated by the Microtubule Deacetylase,**

**HDAC6**

R. A. Joseph,[1] G. T. Kannarkat,[1] C. A. Cheng,[1] D. J. Tuma,[2] P. L. Tuma[1]; [1]Biology, The Catholic University of America, Washington, DC, [2]Liver Study Unit, Veterans Administration Medical Center, Omaha, NE

Chronic alcohol consumption can lead to serious liver disease. Although the disease progression is clinically well-described, the molecular basis for alcohol-induced hepatotoxicity is not well understood. For these studies, WIF-B cells were used to examine hepatocyte specific defects due to ethanol exposure. These cells are an excellent model for studying alcohol-induced hepatotoxicity; they form highly polarized cells in culture and they metabolize alcohol. Previously, we determined microtubules were more highly acetylated and more stable in ethanol-treated WIF-B cells . We found that increased acetylation required ethanol metabolism and was likely mediated by acetaldehyde. Ethanol treatment is also known to impair the trafficking of numerous hepatic proteins. We propose that increased microtubule acetylation and stability may explain the observed impairments. To test this, the trafficking of selected molecules was examined in cells treated with ethanol or specific inhibitors of microtubule deacetylases, i.e., agents that should increase microtubule acetylation in the absence of ethanol. No increased tubulin acetylation was observed in cells treated with nicotinamide, an inhibitor of Sirtuin T2. However, trichostatin (TSA), an inhibitor of histone deacetylase 6 (HDAC6), induced microtubule acetylation in a time- and dose-dependent manner to the same extent as in ethanol-treated cells (~3-fold). The microtubules in TSA-treated cells were more stable and resembled the shorter, more gnarled microtubules that were observed in ethanol-treated cells. In ethanol and TSA-treated cells, the distributions of endocytic organelles were altered and the basolateral accumulation of selected proteins was observed. Although HDAC6 expression levels did not change in ethanol-treated cells, its solubility in permeabilized cells was increased suggesting impaired association with polymeric tubulin. Thus, one possible mechanism to explain microtubule hyperacetylation in ethanol-treated cells is decreased microtubule association of HDAC6 leading to decreased tubulin deacetylation.

1759

**Benomyl Suppresses Microtubule Dynamics, Decreases Tension Across Kinetochore Pairs and Induces Apoptosis in Cancer Cells**

K. Rathinasamy, D. Panda; School of Biosciences and Bioengineering, Indian Institute of Technology, Mumbai, India

Benomyl, an antifungal drug, has been found to arrest mammalian cells at mitosis in parallel with its ability to inhibit cell proliferation. We found that benomyl inhibited microtubule assembly dynamics in mammalian cells, which encouraged us to examine the mechanism by which benomyl arrests mammalian cells at mitosis and induces apoptosis. Cells treated with 5 μM benomyl, a drug concentration that inhibited HeLa cell proliferation by 45 %, arrested HeLa cells at mitosis with nearly normal bipolar spindles with most of the chromosomes aligned at the metaphase plate, and a few chromosomes were scattered near the poles. Cells treated with 20 μM benomyl showed mutipolar mitosis and the chromosomes were not compactly aligned at the metaphase plate. Benomyl (5 μM) decreased intercentrosomal distance in mitotic HeLa cells by 28% from 11.8 ± 1.6 μm to 8.3 ± 1.6 μm and it also perturbed spindle microtubule-kinetochore attachment in the mitotically arrested cells. Benomyl significantly decreased the distance between the sister kinetochore pairs in metaphase cells indicating that the drug impaired the tension across the kinetochores. Further, benomyl induced bcl2 hyper-phosphorylation, reduced the association of bcl2 and bax, and induced apoptosis, which was confirmed by the cleavage of the enzyme poly ADP-ribose polymerase and fragmentation of DNA. Our results showed that benomyl inhibits mitosis by suppressing microtubule dynamics and reducing tension across the kinetochore pairs. Thus, benomyl may be used to study microtubule-mediated alterations in the functions of centrosomes and tension at kinetochores and to understand events leading to apoptosis in tumor cell lines. Supported by Department of Biotechnology, India.

1760

**Microtubule Sliding Powers Proplatelet Elongation from Megakaryocytes during Platelet Production**

S. R. Patel,[1] J. Richardson,[1] H. Schulze,[2] E. Kahle,[1] N. Galjart,[3] K. Drabek,[3] R. Shivdasani,[2] J. H. Hartwig,[1] J. E. Italiano[1]; [1]Hematology, Brigham and Women's Hospital, Boston, MA, [2]Dana Farber Cancer Institute, Boston, MA, [3]Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands

Blood platelet formation is powered by a dramatic reorganization of the microtubule cytoskeleton. During platelet biogenesis, terminally differentiated megakaryocytes use microtubule-based forces to convert their cytoplasm into long psuedopodia, called proplatelets, which remodel into blood platelets. Microtubule bundles line proplatelets and occasionally diverge, forming platelet-sized swellings along the proplatelet shaft. A nascent platelet assembles within the bulbous tip at each proplatelet terminus. Proplatelets elongate at an average rate of 0.85 μm/min. Addition of rhodamine-tubulin to permeabilized proplatelets, immunofluorescence microscopy of the microtubule plus-end marker EB3, and time-lapsed fluorescence microscopy of EB3-GFP expressing megakaryocytes reveal that microtubules continuously polymerize throughout the proplatelet and organize into a bipolar array. EB3-GFP movements indicate that centrosomal nucleation of microtubules predominates in immature megakaryocytes lacking proplatelets, where plus-end growth rates range from 8.9-12.3 μm/min. As megakaryocytes mature and begin to form proplatelets, centrosomal nucleation ceases and microtubule polymerization occurs throughout proplatelets. In contrast to immature megakaryocytes, plus-end growth rates within proplatelets are highly variable, ranging from 1.5-23.5 μm/min. Despite arrest of net microtubule assembly, proplatelets continue to elongate. An alternative mechanism for force generation is microtubule sliding. Upon addition of ATP, Triton X-100 permeabilized proplatelets elongate at a rate of 0.65 μm/min in the absence of microtubule polymerization, indicative of an inherent microtubule sliding mechanism. Immunofluorescence microscopy studies demonstrate that the microtubule-based molecular motor, cytoplasmic dynein, and its regulatory complex, dynactin, are situated along the length of proplatelets and remain after Triton X-100 permeabilization. Furthermore, retroviral expression of dynamitin (p50), which disrupts dynactin-dynein function, inhibits proplatelet elongation. These findings suggest that dynein powers microtubule sliding during proplatelet elongation. We conclude that while continuous microtubule polymerization is necessary to support the enlarging proplatelet mass, the sliding of overlapping microtubules is a vital component of proplatelet elongation.

1761

**Creation of a Computational Model for Cooperativity in Single-stranded Polymers**

A. Bartholomew,[1] R. Ganetzky,[1] P. J. Thomas,[2] L. Romberg[3]; [1]Computer Science, Oberlin College, Oberlin, OH, [2]Mathematics, Oberlin College, Oberlin, OH, [3]Biology, Oberlin College, Oberlin, OH

FtsZ, a bacterial homolog of tubulin, forms single-stranded polymers that assemble cooperatively (Chen et al, 2004). Models for cooperative

polymerization traditionally require polymers to be multistranded, suggesting that new models are now needed. Potentially, cooperativity might emerge if a subunit changes from a low to a high affinity conformation when in contact with adjacent subunits in the polymer. Computer programs that model chemical reactions could determine whether such models for single-stranded polymers produce the lags and critical concentrations indicative of cooperativity. However, conventional programs are difficult to apply to polymers because an unlimited number of different polymer species may exist, whereas these programs require discretely defined species. Additionally, conventional programs cannot efficiently track spatial relationships between subunits within a filament or make rate constants dependent upon them. We are developing a computer program that tracks the behavior of 10,000 subunits based on probabilities of reaction per unit time. The modeling strategy uses a stochastic reaction event generator (Gillespie, 1977). To validate the algorithm, we successfully simulated mathematically solvable polymerization schemes, including isodesmic polymerization and the model for double-stranded polymerization used to fit FtsZ data (Chen et al, 2004). Our program is robust, eliminating feedback loops that can occur in deterministic models in which all long polymers are combined into one chemical class. Additionally, our program successfully simulates polymerization models not easily solved with conventional methods, including systems in which polymers anneal and fragment. We are determining the effects of annealing on polymerization kinetics and polymer length distribution. We are also extending the program to determine whether linking a subunit's reaction rates to the chemical states of its neighbors can explain cooperativity in single-stranded FtsZ polymers.

1762

**Comparative Time Series Analysis of Single Kinetochore Microtubule Dynamics in Yeast**

K. Jaqaman,[1] J. F. Dorn,[1] G. S. Jelson,[2] P. K. Sorger,[2] G. Danuser[1]; [1]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

We want to elucidate the role of kinetochore proteins in regulating kinetochore microtubule (kMT) dynamics and consequently ensuring proper chromosome segregation. We take budding yeast as a model system because of its relative simplicity and powerful genetics, and because in yeast we can track single kMTs by labeling a centromere-proximal region. However, kMT dynamics in yeast are fast and kMT trajectories obtained by 3D time-lapse microscopy suffer from undersampling. Thus the analysis of kMT dynamics is a challenging task, both because of extrinsic stochasticity introduced by undersampling and because of the intrinsic stochasticity of kMT trajectories. Changes in kMT behavior induced by mutations are often comparable in magnitude to the inherent variability of the dynamics in wildtype, even when the mutations are lethal. Therefore, to analyze and compare kMT behavior under different conditions, and to calibrate mathematical models of the molecular-level processes that underlie the observed dynamics, sensitive descriptors that properly characterize kMT trajectories must be employed. Here we propose the use of autoregressive moving average (ARMA) models for that purpose. ARMA models extract the dependence of a stochastic variable on its history and on a related white noise series which embodies the probabilistic nature of the underlying process. ARMA models yield a detailed description of kMT dynamics: In addition to capturing the traditionally employed kMT growth and shrinkage speeds and rescue and catastrophe frequencies, they also reveal the time correlation in kMT trajectories. ARMA descriptors distinguish between G1 kMT behaviors resulting from kinetochore protein mutations, provide the means to distinguish between changes in kMT dynamics due to differential regulation and those due to equilibrium shifts within one regulatory regime, and provide necessary insight for devising mathematical models of the molecular-level processes that underlie the observed kMT dynamics.

1763

**Coordination of Microtubule Employing HVEM of the Adult Retinal Cells in *Drosophila melanogaster***

J. Mun,[1] K. Lee,[1] T. Arii,[2] K. Hama,[2] H. Kwon,[3] D. Studer,[4] S. HAN[1]; [1]Korea University, Seoul, Republic of Korea, [2]National Institute for Physiological Sciences, Okazaki, Japan, [3]Division of Nano-Material and Environmental Science, Daejeon, Republic of Korea, [4]Institute of Anatomy, Bern, Switzerland

The study of structure in *Drosophila melanogaster*'s photoreceptor cells using electron microscopy were carried in detail by Waddington (1962), Farquhar and Palade (1963), and Perry (1968). But, these results can have limitation because of two-dimensional structure. High voltage electron microscopy (HVEM) has been a natural outgrowth of the desire to obtain 3-dimensional information due to problems related to interpretations of 3-dimensional images from 2-dimensional electron microscophs are numerous. In this study, the adult retina of *Drosophila melanogaster* was investigated using fast-freezing, chemical fixation, serial sections, and electron tomogram. The technique of fixation by fast-freezing offers significant advantages over conventional methods. The retinular cell fixed by fast-freezing had smoother cell membranes than that fixed by chemicals. Furthermore, subcellular organelles were preserved well. Therefore we could acquire accurate 3-D models. The distribution of retinular cells, cross-section microtubules and nuclei was observed by their contours of 21 ~ 30 μm, 35 ~ 40 μm, 50 ~ 55 μm, 60 ~ 65 μm, and 73 ~ 79 μm range below distal region. Microtubules are appeared at basal and distal side mainly. And in each range, they existed mainly near mitochondria. After that, the polarity of microtubule in each range of retinal cells was observed. The current data provide us more precise the organization and distribution of microtubule in retinal cell organization in new dimension.

1764

**Thermal Fluctuations of Grafted Microtubules Provide Evidence of a Length Dependent Persistence Length**

F. Pampaloni,[1] G. Lattanzi,[2] A. Jonas,[3] E. Frey,[4] E. Florin[3]; [1]Cell Biology and Biophysics, EMBL, Heidelberg, Germany, [2]Department of Medical Biochemistry, Universita di Bari, Bari, Italy, [3]Center for Nonlinear Dynamics and Department of Physics, University of Texas at Austin, Austin, TX, [4]Department of Physics, Ludwig-Maximilians-Universitat München, Munich, Germany

Microtubules are hollow cylindrical structures that constitute one of the three ma jor classes of cytoskeletal filaments. On the mesoscopic length scale of a cell, their material properties are characterized by a single stiffness parameter, the persistence length Lp . Its value, in general, depends on the microscopic interactions between the constituent globular proteins and the architecture of the microtubule. Here, we use single-particle tracking methods combined with a fluctuation analysis to systematically study the dependence of Lp on the total filament length L. Microtubules are grafted to a substrate with one end free to fluctuate in three dimensions. In living cells, microtubules usually irradiate from a microtubule organizing center toward the cell cortex, hence the boundary conditions adopted in our assay might be close to MT configurations in cells. A fluorescent bead is attached proximally to the free tip and is used to record the thermal fluctuations of the microtubule’s end. The position

distribution functions obtained with this assay allow the precise measurement of Lp for microtubules of different contour length L. Upon varying L between 2.6 and 47.5 μm, we find a systematic sigmoidal increase of Lp from 110 μm to 5035 μm. At the same time we verify that, for a given filament length, the persistence length is constant over the filament within the experimental accuracy. We interpret this length dependence as a consequence of a non-negligible shear deflection determined by subnanometer relative sliding of the protofilaments. Our results may shine new light on the function of microtubules as sophisticated nanometer-sized molecular machines and give a unified explanation of seemingly uncorrelated spreading of microtubules' stiffness previously reported in literature.

1765

### *Toxoplasma gondii*, an Obligate Intracellular Pathgen, Hijacks and Redirects Host Cell Microtubules

J. Hornick, E. Hinchliffe, K. M. Hager; Biological Sciences, University of Notre Dame, Notre Dame, IN

*Toxoplasma gondii* is a pathogen that is profoundly dependent upon the ability to create an intracellular niche safeguarded from host defenses while maintaining accessibility to host nutrients. **We hypothesize that *T. gondii* remodels the host cell cytoskeleton in order to establish an intracellular niche favorable for survival and growth.** *T. gondii* mediates these changes by regulating both host microtubule proteins and host microtubule (MT) transcripts using secreted parasite proteins. Our hypothesis is based on the following electron microscopy, immunofluorescence assay (IFA) and videomicrocopy data. **First**, the parasite actively recruits host cell organelles such as the endoplasmic reticulum and mitochondria to its vacuole membrane. Nocodazole disruption of the host cell MTs decreases the delivery of host mitochondria to the parasite vacuole membrane (PVM). Following drug washout, host MT reform around the PVM. **Second**, infection is correlated with an induction of host microtubule transcription. **Third**, we observe tight MT structures surrounding each PVM. Time-lapse video microscopy of host cells expressing GFP-tubulin reveals that the MTs surrounding each PVM are host-cell derived. **Fourth**, Gamma-tubulin, a MT-organizing center (MTOCs) component, also surrounds each PVM. Gamma-tubulin concentrations are 5-fold higher in infected versus uninfected host cell monolayers. **Fifth**, we observe by videomicroscopy that when superinfected cells undergo mitosis, MTOCs are associated with each of the vacuoles. During exit from mitosis, the MTOCs and the vacuoles themselves coalesce and host cell division is blocked. These observations are important because they suggest both a mechanism for, and the function of, MT remodeling. We conclude that the parasite uses gamma-tubulin as a scaffold to remodel the host cytoskeleton, resulting in the delivery of nutrients and the creation of a stable environment free from the disruptions of cell division.

## Cell Motility III (1766-1793)

1766

### Directing Cell Migration and Proliferation with Engineered Two-Dimensional Spatial Patterns of FGF-2

E. D. Miller,[1] G. W. Fisher,[2] K. Li,[3] L. E. Weiss,[4] L. M. Walker,[5] T. Kanade,[4] P. G. Campbell[6]; [1]Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, [2]Molecular Biosensor and Imaging Center, Carnegie Mellon University, Pittsburgh, PA, [3]Department of Electrical and Computer Engineering, Carnegie Mellon University, Pittsburgh, PA, [4]The Robotics Institute, Carnegie Mellon University, Pittsburgh, PA, [5]Department of Chemical Engineering, Carnegie Mellon University, Pittsburgh, PA, [6]Institute for Complex Engineered Systems, Carnegie Mellon University, Pittsburgh, PA

Spatial patterns of extracellular signaling molecules, hormones, direct cell organizational behavior by providing positional information required for embryonic morphogenesis and post-embryonic tissue repair or regeneration. Engineering arbitrary and persistent spatial hormone patterns to study and control biological patterning relevant to tissue morphogenesis, homeostasis, and repair is vital to understanding tissue development. We have developed an inkjet printing method to create two-dimensional concentration modulated patterns of hormones which direct cellular function including migration and proliferation. We demonstrate this methodology using a model hormone, fibroblast growth factor-2 (FGF-2), naturally immobilized onto a fibrin substrate. Accuracy of the printed patterns was verified using a bioink consisting of native FGF-2 and FGF-2 labeled with a fluorescent dye. Retention of patterned FGF-2 on the fibrin surface *in vitro* was quantified using iodinated FGF-2. Uniform square array patterns and concentration gradients of FGF-2 were printed on fibrin-coated glass slides followed by seeding of the slides with MG-63 human osteosarcoma cells. The cells proliferated in register with the printed patterns demonstrating that the immobilized FGF-2 was biologically active. Cell counts on the patterns indicated that the cells proliferated in a dose-dependent manner up to a level of saturation where increased FGF-2 surface concentrations did not result in increased cell density. Linear concentration gradients and uniform concentration control patterns of FGF-2 were also printed and Swiss3T3 fibroblasts were seeded at the "starting line" at one end of the patterns. The gradient of increasing concentration from the cell source was shown to direct cell migration further than the control patterns with gradient steepness and design influencing the level of cellular response. This growth factor patterning methodology is not limited to FGF-2 and fibrin but is readily extensible to other extracellular matrix substrates and hormones.

1767

### Emergent Properties of the Dendritic Nucleation/Array Treadmilling Model

T. E. Schaus, E. W. Taylor, G. G. Borisy; Cell and Molecular Biology, Northwestern University, Chicago, IL

The dendritic nucleation / array treadmilling model provides a conceptual framework for the actin network driving protrusive cell motility. However, the large number of interacting and self-organizing elements present complexities that defy ready analysis. Such "complex systems" may be studied by computer models to demonstrate emergent properties that are not easily intuited from their component parts. To this end, we have developed a 2-D, stochastic computer model of branching actin polymerization. Sub-models of essential dendritic nucleation mechanisms were incorporated, including discretized actin monomer diffusion, Monte-Carlo filament kinetics, and a bending-resistant flexible plasma membrane. We show that a flexible plasma membrane, while varying over ~100 nm scales, allows the leading edge to conform to network shape and maintain even filament density, but remain remarkably straight over ~1000 nm scales. Despite the flexibility of the leading edge and the stochastic nature of filament polymerization, the model generated a robust self-organization of filaments in which they favored orientations with respect to the leading edge normal of +/- ½ the branch angle. Varying the branch angle did not preclude this development, nor did it affect protrusion rates. Limiting branching of filaments toward the direction of protrusion had a negligible effect on the build-up of backward-facing filament mass or protrusion rates. Protrusion rates were weak functions of the number of free barbed ends within the physiological range of filament densities. Protrusion rates

instead depended critically on work-sharing among polymerizing filaments, a novel aspect of which involves the self-organizing distribution of barbed ends with distance from the leading edge. This permits otherwise thermodynamically-implausible filament extension and has implications for biophysical models of protrusion. We acknowledge funding by the NIH (GM 62431 to GGB) and the Feinberg Pulmonary and Critical Care Division (T32 to TES).

1768

**Probing the Molecular Mechanism in Cell Migration by Cytoskeleton Continuum Mechanics**

L. Ji, J. Lim, K. Hu, C. Waterman-Storer, G. Danuser; Cell Biology, The Scripps Research Institute, La Jolla, CA

Cell movement is mediated by generation of molecular forces acting on the cytoskeleton which by itself undergoes constant assembly and disassembly of actin and microtubule polymers. Whereas many of the molecular constituents of this machinery have been identified on molecule-by-molecule basis, little is understood about how the actions of these many diverse molecular machineries at the system level converge to produce directed motion of whole cells. At the length and time scales of light microscopy, the actin cytoskeleton can be treated as a visco-elastic continuum through which motion-generating forces are transmitted in a highly regulated fashion. To understand how plasticity and changes of the cytoskeleton architecture and the adhesive coupling of the cytoskeleton to the extra-cellular matrix alter cell behavior at the microscopic scale, we established a continuum mechanical model of the actin cytoskeleton mediating protrusive activity at the leading edge of migrating epithelial cells. We developed an algorithm to identify intracellular forces by fitting the partial differential equations describing the relationship between force generation and cytoskeleton deformation to high-resolution of cytoskeleton displacement data sets. These data are extracted from fluorescent speckle microscopy (FSM) images of living cells. We also devised rules to separate contraction forces from adhesion resistance forces. This allows us for the first time to watch the time modulated interplay between contraction and adhesive force distributions inside a cell and to correlate it with the movement of the cell edge. To validate our model, we correlated the reconstructed cellular forces with the spatial distribution of myosin-II and adhesion complexes in multi-spectral FSM of F-actin and myosin or one adhesion protein.

1769

**A Continuum Finite Element Model Predicting Cell Motility**

B. Flaherty, J. P. McGarry, B. P. Murphy, P. E. McHugh; Mechanical and Biomedical Engineering, National University of Ireland, Galway, Galway, Ireland

This research is concerned with the development of a continuum Finite Element (FE) model of a motile cell that includes all major biomechanical and biochemical factors experimentally observed. The development of an FE model to describe a biological process, such as cell motility, represents a major advancement in the use of the finite element method. Constitutive laws, used previously in purely mechanistic applications, will be adapted to include the characteristics seen in a motile cell. The inclusion of biochemical stimuli, which are integral to the generation of forces required for cell movement, represent another major challenge in this work. While a commercially available finite element solver will be used to generate the final results, much work developing code that allows the inclusion of the above constituents will have to be undertaken. The results to date include the generation of accurate cellular geometry from images taken by a confocal microscope. Also a User Material Subroutine (UMAT) was developed. This allows the creation of an active material which in turn generates the asymmetric force that drives a motile cell. Also a User Surface Interaction Subroutine (UINTER) that describes the interfacial behaviour between the cell and substrate has been included. In conclusion, by incorporating the aforementioned components in a two dimensional finite element model of a cell sitting on a substrate, it is possible to model cell movement under the influence of the asymmetric driving force and also mimic the cell/substrate interfacial behaviour, needed to facilitate cell movement.

1770

**Cell Migration in 3D: Sterics, Stiffness and Signals**

M. H. Zaman,[1] L. M. Trapani,[2] A. Siemeski,[1] R. D. Kamm,[1] A. Wells,[3] D. A. Lauffenburger,[1] P. T. Matsudaira[4]; [1]Biological Engineering, MIT, Cambridge, MA, [2]Wellesley College, Wellesley, MA, [3]Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, PA, [4]Whitehead Institute, Cambridge, MA

Most of our current knowledge of cell motility and migration comes from *in vitro* studies carried out on two-dimensional substrates which provide only limited powers of observation. Moving to a 3D environment offers a superior range of observation, however the 3D experiments carried out to date have not studied the effect of matrix properties or cell-matrix interactions on cell migration. In order to fully understand the underlying mechanisms by which cells migrate *in vivo*, it is critical that we study the movement of cells in 3D environments that mimic the properties of the ECM *in vivo*. Using a combination of computer simulations, high resolution imaging and rheology studies, we study the role of adhesion, mechanics, stiffness and matrix proteolysis in cell migration in 3D matrices. Our computational approaches combine novel techniques in ODE based modeling to calculate the forces acting on the cell during migration, Monte Carlo Simulations to study persistence and MD simulations to understand integrin-ECM interactions. Our techniques allow us to quantitatively map the cell migration landscape at various length and time scales. The results from our simulations and experiments show a simultaneous dependence of cell migration behavior on both chemical signals in the ECM and mechanical properties of the cell and the ECM. A complete understanding of this complex process requires that both the mechanics of the matrix and chemical signals be taken into account, as ignoring either of the two key components will lead to incomplete and inaccurate understanding of migration in 3D environments. The novel computational methods developed in our group show good agreement with the experimental studies in predicting the overall behavior of cells migrating in 3D and have provided useful insights in understanding the molecular basis of migration, contact guidance and persistence in 3D environments.

1771

**Identification of Novel Genes that Regulate Cell Motility Using a High Throughput siRNA Screening Approach**

K. J. Simpson, L. M. Selfors, J. Bui, J. S. Brugge; Cell Biology, Harvard Medical School, Boston, MA

Cell migration is a complex process requiring coordinated interactions of many proteins throughout the cell. A comprehensive understanding of the protein networks and their functional relationships requires a broad, system-level analysis of these processes. In conjunction with the Cell

Migration Consortium, we have developed a high throughput assay to screen large collections of siRNAs to identify genes that positively or negatively regulate cell migration. This screen, is based on the well-established wound healing assay for cell motility, employing a robotic pin to generate the wound and automated microscopy to capture the entire wound in a single image. Our screen of 1100 human genes includes all protein phosphatase and kinase family members and a selected set of genes implicated in regulation of cell motility in normal tissues and tumours from diverse origins. We use the mammary epithelial cell line MCF10A and derivatives over-expressing wildtype or constitutively active ErbB2 to identify genes that (a) regulate the response of epithelial cells to a motogen, (b) enhance migration in the "sensitized" background of a non-migratory cell line, and (c) inhibit aberrant cell-autonomous migration of a transformed cell line. Cells are robotically transfected with Dharmacon SMARTpool siRNAs, wounded 52 hours post-transfection, then fixed and stained with rhodamine-phalloidin. Cell viability is determined prior to wounding to assess the impact of the siRNAs on general cell metabolism and proliferation. Motility is scored by the amount of cleared area remaining in the wound space, together with gross cell morphology and actin cytoskeletal alterations. Pilot studies confirm the assay is robust, with migration phenotypes consistent with the literature for RhoA, RhoB and RhoC and Rac1 and Cdc42. We will present preliminary data on the hits from the phosphatase screen that is currently in progress.

1772

**Genome-wide RNAi Analysis Identifies a Network of Proteins Necessary for Caenorhabditis Elegans Distal Tip Cell Migration**

E. J. Cram, H. Shang, J. Schwarzbauer; Molecular Biology, Princeton University, Princeton, NJ

Integrin receptors for extracellular matrix transmit mechanical and biochemical information through molecular connections to the actin cytoskeleton and to a number of intracellular signaling pathways. In *C. elegans*, integrins are essential for embryonic development, muscle cell adhesion and contraction, and migration of nerve cell axons and gonadal distal tip cells. To identify key components involved in integrin signaling and distal tip cell migration in general, we are using an RNA interference (RNAi)-based genetic screen for deformities in gonad morphogenesis caused by aberrant DTC migration. We have surveyed an RNAi library of 16,750 *C. elegans* open reading frames, each contained in a vector optimized for bacterial-mediated RNAi. Of these, 100 clones caused DTC migration defects in > 30% of treated animals. Our analysis indicates that tubulins, other cytoskeletal elements, and signaling proteins are significantly over-represented among the genes isolated in our screen. Using published protein-protein interaction data, we have assembled 38 of these genes into a network. This network has *pat-3*/β integrin as one of its hubs with spokes that link to genes encoding actin, *mig-15*/NIK kinase, the focal adhesion proteins *unc-112* and *unc-35*/talin, and a LIM-domain protein *unc-97*. On the basis of predicted protein interactions within this network, novel candidate genes have been identified and their effects on DTC migration are being determined. This screen has identified a network of interacting proteins that regulate distal tip cell migration, and has identified novel proteins necessary for cell migration in vivo.

1773

**Lateral Membrane Waves Constitute a Universal Dynamic Pattern of Motile Cells**

H. G. Doebereiner,[1,2] B. J. Dubin-Thaler,[1] J. Hofman,[3] H. S. Xenias,[1] T. N. Sims,[4] G. Giannone,[1] M. L. Dustin,[4] C. Wiggins,[3,5] M. P. Sheetz[1]; [1]Biological Sciences, Columbia University, New York, NY, [2]Physics, Columbia University, New York, NY, [3]Applied Physics, Columbia University, New York, NY, [4]Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY, [5]Center for Computational Biology and Bioinformatics, Columbia University, New York, NY

Cell motility is driven by actin polymerization and myosin motor activity. We have monitored active movements of the cell circumference using quantitative DIC and TIRF microscopy. Spreading and motility essays were done on specifically adhesive substrates for a variety of cells including mouse embryonic fibroblasts and T-cells, as well as wing disk cells from drosophila melanogaster. Cultured fibroblasts (RPTPalpha+/+) and wing disk cells (S2R+) were plated on fibronectin and/or vitronectin coated glass slides, respectively. Primary naive T-cells were derived from lymph node and spleen of mice and allowed to adhere on supported bilayers containing ICAM-1 and MHC-peptide complexes. Despite their functional diversity, all those cell types exhibit similar dynamic patterns in their normal membrane velocity. In particular, we found that protrusion and retraction activity is organized in lateral waves running along the cell circumference. We find wave speeds on the order of 100 nm/s. Remarkably, these wave patterns show both spatial and temporal long-range periodic correlations reflecting a corresponding organization of the actomyosin gel.

1774

**Real-time Single Sperm Tracking and Laser Trapping: "Track and Trap"**

L. Shi, E. Botvinick, J. Vinson, M. W. Berns; Bioengineering, University of California, San Diego, La Jolla, CA

Single sperm motility is studied using a novel real-time sperm tracking and laser trapping algorithm. The user is allowed to pick a moving sperm of interest. The algorithm can extract the location of the tracked sperm from a noisy background or when it is out of focus. The stage of the microscope is automatically moved when the tracked sperm is moving toward to the edge of the acquiring screen. The algorithm also has the feature to distinguish the tracked sperm from sperm that it collides with based on the average VCL, traveling distance, and direction of movement after the collision. After enough sperm motility information is collected (length of track), the laser is automatically turned on to trap (capture) the tracked sperm for a predefined time frames. This sperm is continually tracked after the laser is tuned off until the user stops the tracking. Meanwhile, the trajectory, the track speed (VCL) and average VCL of the sperm are saved to the hard disk. The stage movement of the microscope, the image acquisition from the CCD camera and the real-time tracking/trapping algorithm are fast enough to keep the frame rate at 30 frames/second. The fastest sperm tracked in the experiment was 133 microns/second. Unlike other sperm motility software, the real-time single sperm tracking and laser trapping algorithm has no limit on the maximum tracking frames. It can be used not only as a tool to study the effect of laser trapping, but also as an objective and analytical measurement of the sperm motility.

1775

**Statistical Analysis of Phototoxic Effects of Fluorescent Markers on Cell Motility**

W. Timp,[1] S. Hopp,[2] J. G. Evans,[3] P. Matsudaira[2]; [1]Electrical Engineering and Computer Science, MIT, Cambridge, MA, [2]Biology, MIT, Cambridge, MA, [3]Bioimaging Center, MIT, Cambridge, MA

Fluorescent stains are widely used to detect cell proliferation and position, as well as morphology and internal structure. With the advent of new

imaging systems, complete with environmental chambers, it is now possible to image cells over hours, or even days. This long time-lapse data allows us to gather dynamic information about cell motility, but is limited by two problems, photobleaching and phototoxicity. Photobleaching is the gradual fading of the fluorescent dye due to light induced oxidative damage accumulated over time. The far more serious problem is phototoxicity - the effect that these bleached dyes cause in the cell. We wanted to quantitatively determine the effect of phototoxicity on cell motility, comparing different cell types and anti-oxidant additives. It has been suspected for years that these molecules may perturb the cell state - even producing toxic effects, through the combination of intense light and photoreactive species. Effectively, the fluorescent stains are generating free radicals and other oxidative and reactive species after their bombardment with intense light. These free radicals then cause random damage throughout the cell. Different cell types demonstrate different degrees of susceptibility to photodamage. Anti-oxidants are able to ameliorate this effect, allowing the fluorescent stains to be used. We demonstrate this through an analysis of cell speed, calculated using a long term time-lapse scan on a microscope with an environmental chamber. We used brightfield images as a control, assuming that brightfield is the least destructive method of imaging. We then performed the same analysis on the cells with various fluorescent chemical stains, as well as with GFP transfection. Finally, we used the same fluorescent stains in conjunction with anti-oxidants such as Trolox and vitamin C.

1776

**An Automated Phase-contrast Based Live Cell Microscopy Assay of Metastatic Cell Behavior**

D. Loerke,[1] J. De Rooij,[1,2] A. Kerstens,[1] C. Waterman-Storer,[1] G. Danuser[1]; [1]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Department of Cell Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands

The scattering of MDCK cells in response to HGF *in vitro* is a model system that captures key aspects of the transformation of human epithelial cells into metastasizing tumor cells *in vivo*, as cells lose epithelial cell-cell junctions and acquire a motile phenotype. In MDCK cells, both the loss of cell-cell adhesion and the induction of migration occur within 24 hours, which enables us to follow the complete process by live-cell microscopy. For automated analysis of the resulting time-lapse image series, we have developed ScatterTrack, a customized software that automatically detects and tracks individual cells in phase-contrast images. While computationally very demanding for reliable cell detection, the use of phase contrast imaging allows us to analyze long time series by avoiding any phototoxic effects of fluorescence dyes and the signal decay associated with photobleaching. Quantitative analysis of the spatial clustering dynamics of cell distributions allows us to independently evaluate the change in motility and cell-cell adhesion properties, thus separating these two distinct components of the scattering process. In a small-scale drug screen, we have used this software to characterize several pharmacological inhibitors for their capacity to specifically modify either of these components. We find that most described drugs (e.g. SU6656, SB203580) reduce scattering through effects on cell motility rather than cell-cell adhesion, while a minority of inhibitors (e.g. PD98095, PP2) specifically prevent the disruption of cell-cell adhesion. Consequently, selecting drugs on the basis of altered motility alone is not efficient in identifying potent scattering inhibitors. We conclude that our novel live-cell microscopy-based assay allows the differential characterization of the cell-cell adhesion and migration aspects of metastatic cell behavior of HGF-treated MDCK cells, and is a reliable tool for screening new potential pharmacological agents against tumor invasion.

1777

**Viscoelasticity of Actin Networks Growing Under Load**

O. Chaudhuri, S. H. Parekh, D. A. Fletcher; Bioengineering, UCSF/UC Berkeley Joint Graduate Group, University of California at Berkeley and San Francisco; Department of Bioengineering, University of California at Berkeley, Berkeley, CA

Actin filament networks are a major component of eukaryotic cell cytoskeletons that provide mechanical integrity to the cell and drive cell protrusions during crawling motility. The ability of branched actin networks to generate force for motility has been extensively studied with *in vitro* bead motility assays, but the mechanical properties of these networks are not well understood. We reconstituted growing actin networks in a modified atomic force microscope and measured the viscoelastic properties of the branched networks as they grew against increasing loads through stall. Using AFM-based microrheometry, we found that the elasticity of the network increased with increasing load, and we hypothesize that this result is due to an increased number of working filaments at high loads.

1778

**Identification of Novel Tyrosine Substrates in FAK Signaling Pathways**

A. E. Kisailus,[1] E. Shaefer,[2] I. Gelman[1]; [1]Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY, [2]Biosource International, Camarillo, CA

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that plays an essential role in cell migration. Integrin activation by extracellular matrix (ECM) proteins triggers FAK activation via auto-phosphorylation enabling an SH2-dependent interaction with and subsequent activation of the Src tyrosine kinase. The FAK/Src signaling complex plays a role in motility by recruiting and phosphorylating cytoskeletal proteins involved in focal adhesion formation and turnover. A role for FAK in cancer is suggested by its Src-substrate status in tumor cells and by correlations of increased FAK expression/activation levels with increased malignancy. Although a distinctive role for FAK in cell migration, growth, and survival has been implicated, it is unclear whether these functions are encoded by its kinase or protein-scaffolding activities. Furthermore, most FAK substrates identified to date, such as paxillin, CAS, and α-actinin, are also targets of Src phosphorylation. Thus, to identify FAK-specific tyrosine substrates, we used purified, active, recombinant, human FAK to screen a human cDNA expression library. Our *in situ* kinase screen was adapted from previous methods via novel application of a highly sensitive phospho-tyrosine detection system, which used an immunoconjugated infrared-excitable fluor. Controls included overlays with kinase-deficient FAK397F, no kinase (to exclude autophosphorylation by cDNAs encoding tyrosine kinases) and active Src. Using this improved method, we identified 16 new FAK-specific phosphorylation targets. The FAK-dependency of these substrates was verified by immunoprecipitation/anti-phosphotyrosine blotting of FAK+/+ and FAK-/- cell lysates adhered to fibronectin-coated dishes (versus in suspension). The potential substrates are involved in a variety of functions including cell migration, gene transcription, stress response, and metabolism. Study of FAK phosphoryation on these newly identified substrates, such as Trio and RhoGDI2, regulators of Rho-family small GTPases, will further our understanding of how FAK mediates cytoskeletal remodeling and migration.

1779

**Dual Protein Display Technique for Investigating Cell Motility, Cell Adhesion, and Signal Integration**

S. J. Hunt, W. J. Nelson; Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA

Many of the pathways by which extracellular signals provided by cell-cell and cell-matrix adhesion are transduced into changes in intracellular physiology remain to be determined. Particularly lacking in our knowledge of such systems is how the multiple, and often disparate, signals arising from the different forms of cell adhesion are integrated into characteristic cellular responses. An example of such signal integration is found in polarized epithelial cells, which must respond to signaling pathways downstream from both integrin-mediated adhesion to the extracellular matrix (e.g., Rho), and signals arising from E-Cadherin-mediated cell-cell adhesion (e.g., Rac, Cdc42). We have developed a micropatterning technique for displaying two substrates simultaneously on a surface, which we are using to examine the interaction of these two pathways in MDCK cells. We present evidence of signal integration and extinction in our experimental system, as well as examining downstream effects on cellular systems including morphology, actin dynamics, and cell motility.

1780

**Cell Spreading and Migration on Surfaces of Variable Mechanical Stiffness**

R. A. Desai, S. Raghavan, M. T. Yang, C. S. Chen; Bioengineering, University of Pennsylvania, Philadelphia, PA

Initiation of cell migration is crucial to diverse processes such as embryonic development, tissue maintenance and repair, and tumor metastasis. While much is known about the binding interactions between integrins and the extracellular matrix (ECM) that trigger migration, little is known about the role of ECM mechanics in mediating these effects. Here, we investigated how mechanical stiffness of the underlying substrate regulates the transition of cells from a stationary to a migratory state. We developed a surface micropatterning technique in which cells are initially restricted to small adhesive patches surrounded by a nonadhesive background. The nonadhesive regions are then chemically switched to become adhesive, thereby uncaging cells and allowing them to migrate away from prior geometric constraints. We then applied this technique to cells cultured on substrates presenting a bed of vertical cantilevers of different heights. Altering cantilever height enables control over substrate stiffness independent from changes to surface chemistry or ECM presentation to the cell. Preliminary results suggest stiffness of the substrate modulates the ability of cells to develop stress fibers, and initiate cell spreading and migration upon release from micropatterned constraints. We are investigating the interaction between substrate mechanics and cell mechanics, and the subsequent mechanochemical signaling that may underlie these processes. Understanding the mechanochemical phenomena underlying cell-substrate interactions, cell spreading and migration will likely yield a more complete model of how cells probe their physical environment.

1781

**Quantitative Elucidation of a Unique Spatial Gradient Sensing Mechanism in Fibroblasts**

I. Schneider, J. Haugh, E. Parrish; Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC

During wound healing, fibroblast migration is biased by extracellular gradients of platelet-derived growth factor (PDGF). As in other chemotactic cells, such as neutrophils and *Dictyostelium discoideum*, spatial gradient sensing in fibroblasts is mediated by receptor activation of phosphoinositide (PI) 3-kinases and the localized production of 3' PIs in the plasma membrane. However, the responses of these cell types to uniform stimulation suggest fundamental differences in their respective spatial sensing mechanisms. Indeed, while shallow chemoattractant gradients yield robust, "all-or-none" polarization of 3' PI signaling in *Dicty* and neutrophils, we postulated that the gradient sensitivity in fibroblasts depends much more on the average PDGF concentration. Using total internal reflection fluorescence microscopy to quantitatively monitor 3' PI levels in fibroblasts expressing the GFP-AktPH probe, in conjunction with mathematical modeling of the underlying kinetics and spatial 3' PI pattern, we report that fibroblasts possess three distinct regimes of PDGF gradient sensitivity. In response to PDGF gradients with low midpoint concentrations, the 3' PI gradient is roughly proportional to the change in receptor occupancy across the cell. Gradients with intermediate midpoint concentrations, sufficient to recruit most intracellular PI 3-kinase without saturating receptor occupancy, elicit maximal 3' PI gradient sensitivity. At high midpoint concentrations, receptors are saturated, and the response is indistinguishable from that seen after uniform stimulation. We also found that the morphological polarity of the cell imposes an intrinsic bias to the 3' PI pattern. Peripheral, leading-edge regions of the contact area were consistently found to be 'hot spots' of 3' PI lipid concentration, and quantitative analyses revealed that these regions harbor both locally enhanced PI 3-kinase activation and reduced 3' PI turnover. Hot spots enhance or oppose the initial sensing of a PDGF gradient depending on its orientation relative to the cell.

1782

**Distinguishing Models of Eukaryotic Gradient Sensing**

W. Losert,[1] R. Skupsky,[2] R. Nossal,[2] C. Parent,[3] E. Rericha[1]; [1]Dept of Physics, IPST, and IREAP, University of Maryland, College Park, MD, [2]Laboratory of Integrative and Medical Biophysics, NICHD, NIH, Bethesda, MD, [3]NCI, NIH, Bethesda, MD

How can one critically evaluate models of signaling pathways, such as the 3'phosphoinositide pathway for gradient sensing in *Dictyostelium discoideum*, even though there are uncertainties in rate constants and potential interactions with unidentified proteins? We present a modular model of gradient sensing in eukaryotic cells, and new experimental tools to critically test this model. The model describes phosphoinositide (PI)-mediated sensing of cAMP at an intermediate level of detail - we focus on the spatial distribution 3'PIs and a subset of molecules involved in its regulation. We identify two coupled positive feedback loops involving substrate delivery and enzymatic feedback through PI3K and PTEN, and a global inhibitory mechanism through phosphorylation of PI3K. Our analysis indicates that a single signaling pathway can lead to qualitatively different gradient sensing mechanisms. Changing the relative strength of the feedback loops (e.g. by changing concentrations of signaling molecules or values of rate constants) yields four variants with characteristics that resemble different models of gradient sensing in the literature. We find that the model variants may best be distinguished based on the characteristics of the transition from efficient gradient detection to failure. Such transitions to failure occur (1) at low cAMP gradients, and (2) in gradients that change quickly with time. We show new experimental tools to study the transition from efficient gradient detection to failure in single cells: stable small gradients of cAMP of a few percent or less across a cell are generated with a microfluidics device. A new optical micromanipulation technique allows us to generate time dependent gradients by varying the orientation and position of multiple cells in a stationary chemical gradient at speeds of several microns/sec and rotation rates of less than one

rotation/sec. This work was supported by NSF-MRSEC and NIH.

1783

**Modulation of Chemotactic Behavior by Cues from Substrate Topography**

M. M. VanDuijn, D. Fletcher; Dept of Bioengineering, UC Berkeley, Berkeley, CA

Chemotaxis is an important process controlling the migration of cells. It has been the subject of extensive studies, in particular the migration of neutrophils in response to gradients of formylated peptides like fMLP. While such cells are usually studied on a flat substrate, chemotaxis of neutrophils *in vivo* typically involves migration through complex three dimensional tissue. Under these conditions, cells cannot migrate along a straight path along the chemical gradient, but need to circumnavigate other cells and components of the extracellular matrix. Thus, chemical gradients may lead cells in a direction they are physically unable to follow. Here we investigate the interplay of structural and chemical cues on the navigation of chemotactic cells. HL-60 cells were differentiated with DMSO to until they showed neutrophils-like chemotactic properties, and were then allowed to migrate on coverslips decorated with paths and obstacles that were microfabricated from SU8. A chemotactic gradient was delivered from a micropipette with a constant backpressure, and cell movements were tracked. The data suggest that structural features on the substrate can override cues from the chemical gradient, leading to cells crawling on a tangent or even away from a source of fMLP. This shows that cells are not stringently directed by the local chemical gradient, but can combine different sources of information to guide their movement.

1784

**Biochemical Mechanics of Nematode Spermatozoon Crawling**

M. Zajac, C. W. Wolgemuth; Cell Biology, UConn Health Center, Farmington, CT

Cell crawling requires three main processes: polymerization at the leading edge, adhesion to the substrate, and retraction at the rear. The objective of this work is an extensible model to describe the biochemical regulation of the cytoskeletal deformation and reorganization that drives motility in crawling cells. Recent experiments using nematode spermatozoa and spermatozoon extracts reveal that the Major Sperm Protein (MSP) cytoskeleton in these cells drives locomotion in a similar fashion as has been observed in the more familiar translocation of actin-based cells. However, the MSP system is simpler and therefore more amenable to modeling. Our simulation method uses two-phase gel mechanics which treats the cytoskeleton as a deformable elastic meshwork and also accounts for the fluid motion of the cytosol. The thermodynamic underpinnings of this model capture elastic, ionic, entropic, electrostatic and hydrophobic interactions between cytosolic and cytoskeletal matter. Explicit treatment of cytosolic flow allows competition or collaboration between advection and diffusion as mechanism for intracellular transport. Level set methods facilitate robust treatment of moving cell boundaries where cytoskeletal assembly converts between the liquid (cytosolic) and solid (cytoskeletal) phases as monomers enter or leave solution and also provide an algorithm for computing lateral diffusion along the deforming cell membrane, thereby enabling the simulation of cytoskeletal nucleation by membrane bound proteins. Simulation results realistically predict the polymerization rate at the leading edge of the cell and the force generated by the deforming cytoskeleton and illustrate the potentially catastrophic feedback between changing cell geometry and intracellular processes, both chemical and physical. In conclusion, initial simulations suggest that gel mechanics and level set methods may provide an adaptable basis for wholistic reconstruction of cell motility, starting from experimental measurements of individual components.

1785

***Spiroplasma* Swim by a Processive Change in Body Helicity**

J. W. Shaevitz,[1] J. Y. Lee,[2] D. A. Fletcher[3]; [1]Integrative Biology, U.C. Berkeley, Berkeley, CA, [2]Plant and Microbial Biology, U.C. Berkeley, Berkeley, CA, [3]Bioengineering, U.C. Berkeley, Berkeley, CA

Microscopic organisms must rely on very different strategies than their macroscopic counterparts to swim through liquid. To date, the best understood method for prokaryotic swimming employs the rotation of flagella. Here, we show that *Spiroplasma melliferum*, tiny helical bacteria that infect plants and insects, use a very different approach. By measuring cell kinematics during free swimming, we find that propulsion is generated by the propagation of kink pairs down the length of the cell body. A processive change in the helicity of the body creates these waves and enables directional movement in a viscous environment.

1786

**Transient Adhesion-mediated PKA Activation at the Leading Edge of Migrating Cells**

L. E. Goldfinger,[1] K. H. Kain,[1] J. Zhang,[2] R. Y. Tsien,[3] M. H. Ginsberg[1]; [1]Medicine, University of California, San Diego, La Jolla, CA, [2]Department of Pharmacology and Molecular Sciences and Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, [3]Departments of Pharmacology and Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA

cAMP-dependent protein kinase A (PKA) is important in directional cell migration and axonal pathfinding. Activation and deactivation of endogenous PKA can be imaged by fluorescence resonance energy transfer within AKAR2.2, comprising monomeric cyan and yellow fluorescent proteins linked by a pThr-binding domain and a Thr-containing PKA substrate. When targeted to the plasma membrane, AKAR2.2 revealed transient integrin-mediated activation of PKA at the leading edge of migrating CHO cells. Leading edge PKA activation was seen with two different integrins and was required for efficient forward protrusion at the cell front. PI3-kinase has been proposed as the initiator of polarization of migrating cells; however, polarized PKA activation was PI3-kinase-independent and is therefore an autonomous, early, and critical directional signal in cell migration. Thus we describe a novel, compartment-specific PKA activation biosensor and utilize it to identify a new, PI3-kinase-independent, cellular compass.

1787

**Integrin α6β4 Promotes Expression of Autotaxin/ENPP2 Autocrine Motility Factor**

M. Chen, K. O'Connor; Department of Surgery and the Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, TX

In advanced breast carcinomas, the α6β4 integrin is associated with a migratory and invasive phenotype. In our current study, we show that expression of the α6β4 integrin in MDA-MB-435 breast carcinoma cells leads to increased expression of the autocrine motility factor autotaxin, as determined by Affymetrix gene chip, real time quantitative RT-PCR and immunoblot analyses. We further demonstrate that increased autotaxin

secretion from integrin α6β4 expressing cells acts to enhance chemotaxis through its ability to convert lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA). We determine that integrin α6β4-dependent overexpression of autotaxin in MDA-MB-435 cells is mediated by NFAT1, but not NFAT5, through the use of siRNAs that specifically target autotaxin, integrin β4, NFAT1 and NFAT5. Finally, we show by electrophoretic mobility shift assays that three consensus NFAT binding site found in the autotaxin promotor specifically binds NFAT1 from integrin α6β4 expressing cells and is sensitive to calcineurin inhibition. In summary, we find that the α6β4 integrin potentiates autotaxin expression through the upregulation and activation of NFAT1. These observations highlight for the first time a mechanism by which NFAT transcription factors can facilitate an invasive and motile phenotype downstream of integrin α6β4 signaling.

1788

**Myosin Activity is Required for Symmetry Breaking and Motility Initiation in Keratocytes**

P. T. Yam,[1] J. A. Theriot[2]; [1]Biochemistry, Stanford University School of Medicine, Stanford University, CA, [2]Biochemistry and Microbiology and Immunology, Stanford University School of Medicine, Stanford University, CA

We have observed the spontaneous initiation of actin-based motility in keratocytes, a rapidly moving fish epithelial cell. This motility initiation was accompanied by changes in the symmetry (from radial to bilateral), shape, and cytoskeletal dynamics of the cell. Individual stationary keratocytes were circular, while motile keratocytes had a half-moon shape. We have found that the F-actin meshwork in stationary keratocytes flows centripetally from the leading edge to the cell body, in contrast to motile keratocytes, where the F-actin meshwork remains mostly stationary with respect to the substrate as the cells move. Over time, circular stationary keratocytes were observed to break symmetry spontaneously and generate a well-defined and persistent structural polarity in the absence of extrinsic spatial cues. We wished to determine whether spontaneous polarity was generated by specification of the leading edge (as in chemotaxis) or specification of the trailing edge. Time-lapse phase contrast microscopy indicated that during spontaneous motility initiation the rear of the cell retracted first, followed by forward translocation of the cell body and finally the front cell margin, over a period of ~200-400 seconds. Rear retraction was accompanied by a decrease in lamellipodial thickness at the rear, and an increase in the rate of centripetal actin flow at the rear, suggesting that spontaneous motility initiation in keratocytes first involves changes in the rear of the cell. Depolymerization of microtubules with nocodazole did not inhibit motility initiation. Inhibition of myosin II with blebbistatin decreased the frequency of motility initiation. The blebbistatin phenotype was mimicked by the Rho kinase inhibitor Y-27632 but not the myosin light chain kinase inhibitor ML-7, suggesting that an increase in actin network contractility at the rear of the cell regulated by myosin and Rho kinase is important for symmetry breaking.

1789

**Local Application of PDGF Induces Rapid Spreading and Tractional Force Generation by Isolated Cells Inside 3-D Collagen Matrices**

W. M. Petroll, L. Ma; Ophthalmology, University of Texas Southwestern Medical Center, Dallas, TX

We have previously studied cell-matrix mechanical interactions within 3-D collagen matrices using high magnification time-lapse imaging. The goal of this study was to extend this model to allow local application of growth factors or other reagents near isolated cells. Human corneal fibroblasts were plated at low density inside 100 μm thick fibrillar collagen matrices and cultured for 1 to 2 days in serum-free media. Following 1 hour of time-lapse DIC imaging, a glass microneedle (Femtotip) loaded with either PDGF or vehicle control solution was inserted axially into the ECM, approximately 20 μm from the leading edge of an isolated cell of interest. The loaded solution was then injected into the matrix, and time-lapse imaging was continued for another 1-2 hours. Changes in cell morphology and extracellular matrix (ECM) deformation were assessed using MetaMorph. Insertion of microneedles was generally achieved without significant disruption or deformation of the ECM. Cells randomly extended and retracted cell processes both before and after needle insertion; similar behavior was observed following control injections. In contrast, injection of PDGF induced rapid cell spreading toward the microneedle tip, with both extension of existing pseudopodia and formation of new processes. Tractional force was generated during PDGF-induced spreading, as indicated by pulling in of individual collagen fibrils in front of pseudopodia. Overall, the ability to apply reagents adjacent to individual cells should be a useful tool for studying cell motility within 3-D matrices. In this study, rapid cell spreading and tractional force generation was demonstrated in response to PDGF, presumably through upregulation of Rac. By moving the needle laterally following insertion, the additional effects of local mechanical perturbation on cell behavior could potentially be assessed using this system.

1790

**Comparison of Light-induced Direction Change in Three Diatom Species**

S. A. Cohn, K. R. Monroe, M. P. Wemhoff; Biological Sciences, DePaul University, Chicago, IL

Previous work from our lab indicated that diatom motility is light sensitive, with the direction of cell movement being responsive to light irradiance at the tips of the cells. Exposure to high irradiance light at cell tips can induce a reversal of cell direction (*Diat. Res.* **19**:167) and several different diatom species, including *Craticula cuspidata*, *Stauroneis phoenicenteron*, and *Pinnularia viridis* (*J. Phycol.* **32**:928) have shown a characteristic spectral sensitivity with respect to light attraction. Our current work has investigated these three species for their spectral sensitivity to direction changes induced by high-irradiance light. The leading ends of moving cells were irradiated with high irradiance light ($\geq 10^5$ μmol/m$^2$sec) at either 470, 550, or 650 nm (blue, green or red light, respectively), and observed to determine the time it took for cells to change direction. We found that each species displayed different spectral sensitivities. For example, using 1 sec irradiations, *Craticula* cells were about equally sensitive to blue and green light (changing direction 11±1 and 15±4 sec after irradiation, respectively), while irradiations with red light showed no significant response compared to unirradiated control cells (changing direction 44±6 and 56±6 sec after irradiation, respectively). In contrast, *Stauroneis* cells were responsive to all three light wavelengths, showing greatest sensitivity to blue and red light. *Pinnularia* cells were only responsive to blue light, showing no significant sensitivity to either green or red light. These spectral sensitivities correlate well with those seen for the attraction of these cells into light at low irradiance, suggesting that the low light (into the light) and high light (away from the light) responses are using the same light detection mechanism. This work was supported by DePaul University Research Council, College of Liberal Arts & Sciences, and NSF Grant IBN-9982897.

1791

**A Nocodazole-Sensitive Novel Immune Cell in the Sea Urchin, *Lytichinus variegatus*: Increased Abundance during Spawning**

J. R. Cahlander, B. M. Johnson, S. Amy, L. Winslow; Biology, Northwestern College, St. Paul, MN

Coelomic fluid of the sea urchin *Lytichinus variegatus* has been reported to contain petalloid, red spherule, colorless spherule and migratory coelomocytes. We observed a cell type, typically low in abundance, that increased significantly during the sea urchin's spawning seasons. The level of coelomic fluid and overall number of immune cells during spawning decreases since the coelomic cavity is consumed with swollen gonads containing gametes. Cell counts were performed from September through May, including two spawning seasons (November and March). Our results showed a 50% increase in the unidentified cell type during spawning seasons while ratios of all other coelomocytes remained constant. After spawning, the numbers of these cells precipitously dropped. The cells show a striking resemblance to red spherule coelomocytes in both morphology and motility but are not pigmented. The cells appear multi-lobed, extend and retract blebs in three dimensions but do not appear to translocate linearly. Using digital-enhanced video microscopy, the rate of motility and cell surface bleb formation showed accelerated and more fluid movements than those observed in the red spherule cells. Bleb formation was demonstrated to be dependent on the acto-myosin cytoskeleton based on perfusion experiments using phalloidin, cytochalasin B and blebbistatin. The microtubule disruption drug, taxol, had no effect on cell shape or motility. However, cells treated with the microtubule disruption drug, nocodazole, lysed within seconds of exposure. We thus refer to these cells as Nocodazole Sensitive Cells or NSCs. The unique function of NSCs in sea urchin immunity is presently unknown, but may indicate a complex mechanism of sea urchin immunocompetence where a specific immune defense, needed during sea urchin spawning, is accomplished by NSCs.

1792

***cnr15*, a *Dictyostelium* Cell Number Regulation Gene, is Required for Size Regulation Mediated by Counting Factor**

Y. Tang,[1] T. Gao,[2] D. Hatton,[2] R. Gomer[2,1]; [1]Department of Biochemistry & Cell Biology, Rice University, Houston, TX, [2]Howard Hughes Medical Institute, Houston, TX

Unicellular *Dictyostelium* cells aggregate to form multicellular fruiting bodies during development triggered by the lack of food. Counting factor (CF), a secreted protein complex, was previously found to regulate aggregate size. High levels of CF induce the formation of small aggregates and low levels of CF cause the formation of big aggregates. To understand the molecular mechanisms of CF's regulation of aggregate size, a second-site suppressor screen was conducted to find the components of the CF signal transduction pathway. *cnr15*, one of the cell number regulation (cnr) genes identified in this screen, was found to affect aggregate size. *cnr15* is predicted to encode a Ser/Thr and Tyr dual specificity phosphatase, sharing 40% identity to *Dictyostelium* 3'-phosphoinositide phosphatase known as PTEN. *cnr15*$^-$ cells, generated by homologous recombination in wild-type Ax2 cells, formed smaller fruiting bodies in comparison with those formed by Ax2 cells. *cnr15*$^-$ cells exhibited a higher random cell motility and a slightly lower cell-cell adhesion than Ax2 cells do, which is consistent with previous observations that higher random cell motility and lower cell-cell adhesion lead to smaller aggregates in *Dictyostelium*. *cnr15*$^-$ cells showed a higher level of polymerized actin and a lower level of assembled myosin II in response to cAMP stimulation, indicating Cnr15 regulates cell motility via regulating cytoskeleton proteins. *cnr15*$^-$ cells were insensitive to addition or depletion of CF, indicating that loss of *cnr15* abrogates CF's size regulation effects. Our results suggested that Cnr15 is required for *Dictyostelium* aggregate size regulation mediated by CF.

1793

**HEI10, E3 Ubiquitin ligase, Negatively Regulates Cell Motility**

M. K. Singh,[1] E. Nicolas,[1] W. Gherraby,[1] D. Dadke,[1] S. R. Lessin,[2] E. A. Golemis[1]; [1]Basic Sciences, Fox Chase Cancer Center, Philadelphia, PA, [2]Medical Sciences, Fox Chase Cancer Center, Philadelphia, PA

HEI10 defines a novel class of RING finger protein that regulates the passage through G2. Previously, we have shown that HEI10 functions as an E3 ubiquitin ligase to promote the proteolysis of cyclin B, such that overexpression of HEI10 extends the G2 phase of cell cycle. HEI10 is itself subject to cell-cycle-controlled changes in expression and localization, and is an *in vitro* substrate for cyclin B/cdc2, implying a feedback circuit between the HEI10 protein and the cell cycle machinery. Given that HEI10 localizes in part to focal adhesions, and the HEI10 mRNA has been reported to be particularly abundant in aggressive melanomas, we wished to delineate the functional role of HEI10 in cancer with following aims: 1) Does depletion of HEI10 cause changes in cell migration and attachment, compatible with a role in metastasis, and 2) Is HEI10 more abundant in aggressive versus non-aggressive tumors, and if so, is this difference specific to melanoma? Using live cell imaging and Boyden chamber assays, we have found that siRNA-mediated depletion of HEI10 increases cell motility. HEI10 depleted cells spread over a greater area than control-depleted cells, although the rate of cell reattachment following trypsinization was not altered. HEI10-depleted cells also ceased to proliferate, with a reduced number of cells observed in G2. HEI10 depletion did not affect cellular invasion through Matrigel. Similar results were obtained in both the U2OS (osteosarcoma), and MCF7 (breast adenocarcinoma) cell lines. These data indicate that HEI10 negatively regulates cell motility, perhaps through promoting the degradation of a pro-motility signaling protein. Separately, we have found that HEI10 is not particularly abundant in malignant melanomas, but is well-expressed in many primary tissues and cell lines. Investigation of the mechanism of HEI10 action in control of cell migration is in progress.

## Cytoskeleton-Membrane Interactions II (1794-1807)

1794

**Genetic Evidence that the PH Domain of Beta Spectrin is Responsible for Spectrin Targeting**

A. Das, C. Base, R. Dubreuil; Biological Sciences, Univ. of Ill. Chicago, Chicago, IL

We used a transgene replacement strategy to examine the contributions of the putative ankyrin binding domain and the pleckstrin homology (PH) domain to β spectrin function in the *Drosophila* midgut epithelium. A control myc-epitope-tagged wild type transgene rescued a lethal β spectrin mutation and the myc-tagged protein was correctly targeted in midgut copper cells. Transgenic β spectrin colocalized with endogenous α spectrin at the septate junction and in the basolateral plasma membrane domain below the level of the septate junction, but not in the apical domain. One mutant transgene in which the putative ankyrin binding repeat domain was replaced with an irrelevant repeat domain from α spectrin was also

targeted correctly in copper cells and efficiently rescued mutants to adulthood. This result indicates that either the ankyrin binding site is located elsewhere in β spectrin or that ankyrin binding is dispensible for spectrin function. In contrast, a truncated β spectrin transgene lacking the C-terminal PH domain (ΔPH) was not correctly targeted in copper cells and it exhibited a poor efficiency of mutant rescue. Interestingly the ΔPH protein was targeted only to the septate junction in a wild type background and even that targeting activity was lost in the absence of wild type β spectrin. The results suggest that there are two different mechanisms operating in copper cells to target spectrin to the septate junction and to the subjunctional basolateral domain. Remarkably, a very small number of ΔPH-spectrin-expressing mutants were rescued to adulthood. These flies appeared normal except that they were approximately half the size of their wild type siblings. Mutants without the ΔPH transgene always died before larval hatching. Thus there are additional essential functions of spectrin that are independent of the PH domain.

1795

**CAMSAP1 - A Calcium/Calmodulin-Regulated Spectrin-Associated Protein That is Required for Neurite Outgrowth from PC12 Cells**

M. D. A. King,[1] G. W. Phillips,[1] P. A. Bignone,[1] P. M. Taylor-Harris,[2] A. M. Maggs,[2] J. C. Pinder,[2] M. J. Allen,[1] A. J. Baines[1]; [1]Biosciences, University of Kent, Canterbury, United Kingdom, [2]Randall Division of Cell and Molecular Biophysics, King's College, London, United Kingdom

We describe CAMSAP1, a calmodulin-regulated spectrin-associated protein. The human CAMSAP1 gene is on chromosome 9 at q34.3. In vitro, CAMSAP1 binds $Ca^{2+}$/calmodulin and brain spectrin. The binding site for CAMSAP1 in spectrin is located in betaII-sigma1-spectrin within the linker between the last triple helical repeat and the pleckstrin homology domain. The splice variant betaII-sigma2, that shares only part of this region, does not bind CAMSAP1 in vitro. Native complexes of betaII-sigma1-containing spectrin co-immunoprecipitate with CAMSAP1 from rat brain extracts in EGTA. $Ca^{2+}$ inhibited immunoprecipitation but in $Ca^{2+}$/trifluoperazine, immunoprecipitation was restored indicating that calmodulin regulates CAMSAP1-spectrin interaction. Immunofluorescence on PC12 neurons demonstrated CAMSAP1 in cell bodies, neurites and growth cones in close association with spectrin. To investigate the function of CAMSAP1 in PC12 cells, we knocked down its expression with siRNA. 24 hours after siRNA transfection, CAMSAP1 was undetectable by immunofluorescence, but unchanged in controls. Importantly, knocked-down cells did not respond to NGF by extending neurites, even though ERK was activated. CAMSAP1 evidently has a role in adult brain too: it is abundant in cerebellar granule neurons. CAMSAP1 is one of three related genes in vertebrates; single copies exist in the genomes of invertebrate animals. All these proteins contain a calponin homology domain and a novel C-terminal domain. We have extended our analysis of CAMSAP1 by investigating the Drosophila homolog (dCAMSAP). Two alleles containing P-element insertions in the dCAMSAP gene are embryonic recessive lethal at approximately stage 16; the trans-heterozygotes are also lethal. They show severe defects in both the peripheral and central nervous systems. We conclude that CAMSAP1 plays a direct role in formation of the nervous system, and that it links the established spectrin and calmodulin pathways in this process downstream of ERK.

1796

**Isoform Specificity of Ankyrin-B: A Site in the Divergent C-Terminal Domain is Required for Intramolecular Association**

K. M. Abdi,[1] J. Q. Davis,[1] P. J. Mohler,[2] V. Bennett[1]; [1]Cell Biology, Duke University, Durham, NC, [2]Department of Pathology, Vanderbilt University, Nashville, TN

Ankyrins provide a direct link for integral membrane proteins to the underlying spectrin/actin cytoskeleton. The three major ankyrin gene products, ankyrins R, B, and G contain significant amino acid identity and are co-expressed in many cell types, but maintain unique functions *in vivo*. Recent studies have identified the highly divergent C-terminal regulatory domain in ankyrin-B as the key domain for driving ankyrin-B-specific functions in mouse neonatal cardiomyocytes. The structural basis for this C-terminal domain driven specificity was evaluated in this study. Here we describe an intramolecular interaction between the C-terminal domain and the membrane-binding domain of ankyrin-B using pure proteins in solution and the yeast two-hybrid assay. Through extensive deletion and alanine scanning mutagenesis, we have mapped key residues for interaction in both domains. Amino acids 1597EED1599 located in the ankyrin-B C-terminal domain are necessary for interaction with the membrane-binding domain and are required for localization of InsP3-receptors in cardiomyocytes. Amino acids 37R/40R located in the first ankyrin repeats of the membrane-binding domain are also required for interaction in yeast two-hybrid assays. Physical properties of the ankyrin-B C-terminal domain, determined by circular dichroism spectroscopy and hydrodynamic parameters, reveal it is unstructured and highly extended in solution. Similar structural studies performed on full-length 220 kDa ankyrin-B harboring alanine substitutions, 1597AAA1599, reveal an increased frictional ratio when compared to wild type ankyrin-B. Taken together these results suggest a model of an extended and unstructured C-terminal domain folding back to bind and regulate the membrane-binding domain of ankyrin-B. They also provide a mechanism of intramolecular interaction as a means to drive ankyrin-B functional specificity in-vivo.

1797

**Study of the Effect of Bradykinin and Thrombin on the Cytoskeleton by Atomic Force Microscopy**

C. Cuerrier, F. Gobeil, M. Grandbois; Department of Pharmacologie, Université de Sherbrooke, Sherbrooke, PQ, Canada

Thrombin (TB) and Bradykinin (BK) are important in the maintenance of vascular homeostasis. Indeed, their cellular effects induce important physiologic responses by activating specific receptors (PAR-1 and $B_2$) that induce intracellular signaling and lead to cytoskeletal reorganization. In the present study, we have quantified the effects of these agonists on cytoskeletal reorganization, by measuring the interaction forces between the cell membrane and cytoskeleton by atomic force microscopy (AFM) and by fluorescent microscopy using a phalloidin probe. Membrane tethers (MT) can be extracted from individual HUVEC cells by the establishment of physical contact between the cell and an AFM cantilever. Retraction of the cantilever induces MT formation, characterized by a constant elongation force. These force measurements can be used to monitor the cytoskeletal reorganization induced by BK and TB signaling pathways. Time course analysis of TB (10 nM) and BK (1 µM) activities indicate a significant increase in elongation forces (47 and 51 pN, respectively) after 30 to 45 minutes in comparison to the control (39 pN), suggesting an increase in membrane-cytoskeleton interaction and clearly indicating an important biomechanical response of the cell to these treatments. These AFM results on the TB and BK - induced cytoskeletal reorganization were confirmed by fluorescence imaging. Our results show that it is possible to mechanically and temporally quantify structural changes in the cytoskeleton by AFM. This new experimental model could be used to study the implication of cytoskeleton reorganization in physiological processes such as endothelium permeability and cell motility.

1798

**Vinculin is Activated by Coordinated Action of Talin and F-actin**

H. Chen, D. Choudhury, S. W. Craig; Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD

Published data demonstrate that vinculin is maintained in its high affinity autoinhibited state by cooperation between two lower affinity intramolecular interfaces, D1/Vt and D4/Vt. This finding, together with the inability of talin rod domain to bind full length vinculin in solution, suggest a model in which two ligands cooperate to activate vinculin by disrupting both intramolecular interfaces. However, crystal structures of D1 and VBS-3, a talin binding peptide, and of the 4-helix domain of talin that contains VBS-3, indicate that talin itself must undergo activation to present the VBS-3 peptide to vinculin. Once activated, talin alone may be capable of activating vinculin. To discriminate between these models we first examined the solution binding of TAMRA-labeled VBS-3 peptide to D1, D1-D4 (Vh), and vinculin. Whereas VBS3 binds to D1 and Vh with ~100nM affinity, binding to native vinculin was undetectable. We conclude that the VBS-3 binding site on autoinhibited vinculin is kinetically inaccessible. We used a new vinculin FRET probe to assay the ability of ligands to activate vinculin. Neither F-actin, a ligand for vinculin tail domain, nor talin rod domain, a ligand for D1, were able to activate the FRET probe in lysates of HEK293 cells. However, in the presence of 5uM F-actin, talin rod domain (0-2uM) induced dose-dependent activation of the vinculin FRET probe. This and other data lead us to propose that ligands for D1 may bind to vinculin when Vt momentarily dissociates from D1, but are quickly displaced by Vt unless Vt is bound by another ligand or otherwise modified. Our findings support a dual-ligand activation model, and also suggest a mechanism for controlling how long vinculin remains activated.

1799

**Suramin Effects on *Trypanosoma cruzi*: Cytochemical and Immunocytochemical Evidences of Changes on the Flagellum-Cell Body Adhesion Region**

D. F. R. Bisaggio,[1] L. Campanati,[2] R. C. V. Pinto,[1] T. Souto-Padrón[1]; [1]Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Suramin has been previously reported to inhibit distinct cell enzymes besides the effects on synthesis and distribution of cytoskeleton proteins. Our study indicates that long lasting incubation of *Trypanosoma cruzi* in the presence of suramin caused a partial or complete detachment of the flagellum from the cell body besides an accentuated decrease on parasite motility. Immunofluorescence analysis of the region of adhesion between the cell body and the flagellum on suramin treated trypomastigotes did not show any difference in the localization of FAZ antigens recognized by 4D9 and L3B2 monoclonal antibodies despite the presence of a detached flagellum. On the other hand, suramin caused a significant change on the surface expression of FRA antigen, which was observed throughout the surface of trypomastigotes. Cytochemical localization of cationized ferritin showed that anionic particles gain access to the space between the cell and flagellar membranes as well as to the flagellar pocket in suramin treated parasites indicating alterations on extracellular components of the region of adhesion between the cell body and the flagellum.

1800

**A Novel Microtubule Binding and Bundling Domain in a Coiled-coil ER Protein, p180/Ribosome Receptor**

K. Ogawa-Goto,[1,2,3] T. Ueno,[1] K. Tanaka,[1] K. Tanaka,[2] T. Sata,[2] S. Irie[1,3]; [1]Nippi Research Institute of Biomatrix, Tokyo, Japan, [2]NIID, Tokyo, Japan, [3]Japan Institute of Leather Research, Tokyo, Japan

**Introduction**: The ER uses microtubules (MT) as a framework for extending its reticular network, while mechanism by which the ER maintains this structure in vivo remains unclear. Here we present data that 180/ribosome receptor, a coiled-coil protein on the ER membrane, has novel MT binding domains, and could contribute spatial positioning of the ER along the MT. **Results**: As overexpression of p180 modified the MT arrays in cultured cells, we determined whether p180 binding to MT is direct and which domain is responsible for this interaction. By *in vitro* co-sedimentation assays, we identified a novel MT-binding domain in the coiled-coil region of p180, which induced MT bundling both in vitro and in vivo. Bacterially expressed full-length p180 interacted with MT efficiently. To address a possible role of p180 on the ER organization, we examined siRNA treatment of 180 in human diploid fibroblasts containing abundant endogenous p180. Depletion of p180 is sufficient to alter their normal ER distribution to retract around the nucleus, while expression levels of other ER markers were not changed. Differing degrees of p180 depletion were coincidentally associated with severity of the ER retraction. In these si-RNA transfected cells, re-distribution of MTs occurred. **Conclusions**: Our data indicate that p180 could function as a direct linker between the ER and MT, and seems to affect their spatial distributions in the cultured cells.

1801

**Tubulin Detyrosination Affects Microtubule Tips Protein Complex: Implication in Spindle Position and Neuronal Organisation**

L. Peris,[1] A. Andrieux,[1] M. Thery,[2] J. Fauré,[1] J. Wehland,[3] M. Bornens,[2] D. Job[1]; [1]Rhone Alpes, DRDC-CS CEA INSERM U366, Grenoble, France, [2]Institut Curie UMR144 CNRS, Paris, France, [3]German Research Center of Biotechnology, Braunschweig, Germany

Tubulin is subject to a special cycle of detyrosination/tyrosination in which the C-terminal tyrosine of α-tubulin is cyclically removed by a carboxypeptidase and readded by a tubulin-tyrosine-ligase (TTL). This tyrosination cycle is conserved in evolution, yet its physiological importance is a matter of conjecture. TTL suppression in mice causes perinatal death due to severe brain disorganization. Spindle positioning in neuronal progenitors was perturbed with resulting imbalance of proliferation/differentiation events. In addition, mitotic spindle orientation was also altered in cultured TTL null fibroblasts grown in special restrictive micropatterns. In culture, TTL null neurons display morphogenetic anomalies including an accelerated and erratic time course of neurite outgrowth and a premature axonal differentiation. CLIP family proteins (CLIP170, CLIP 115 and p150Glued), which links microtubule ends to the cell cortex, failed to associate with microtubule tips in TTL null cells and showed a reduced interaction with detyrosinated microtubules *in vitro.* We propose that the tyrosination cycle is required for the control of microtubule interactions with the cell cortex and is thereby critical for mitotic spindle orientation and neurogenesis.

1802

**Mitotic Spindle Oscillations**

S. W. Grill,[1,2,3] K. Kruse,[2] J. C. Roeper,[3] J. Pecreaux,[3] A. A. Hyman,[3] J. Howard,[3] F. Jülicher[2]; [1]Physics, University of California, Berkeley, CA,

[2]Max Planck Institute for the Physics of Complex Systems, Dresden, Germany, [3]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

During unequal cell division the mitotic spindle is positioned away from the center of the cell before cell cleavage, which in many biological systems is accompanied by oscillatory movement of the spindle. We present a simple theoretical description for mitotic spindle oscillations. We show that the cooperative attachment and detachment of cortical force generators to astral microtubules generally leads to spontaneous oscillations beyond a critical number of force generators. This mechanism can quantitatively describe the spindle oscillations observed during unequal division of the one cell stage Caenorhabditis elegans embryo. Finally, we measure the oscillation threshold by RNA mediated interference of the G-protein regulator gpr-1/2.

1803

**Cytoskeletal Dynamics Influence S100A8 and S100A9 Localization and Movement**

A. Broome, R. L. Eckert; Physiology & Biophysics, Case Western Reserve University, Cleveland, OH

Epidermal hyperproliferation, premature cell death, and epidermal remodeling lead to a psoriatic architecture. As proliferative keratinocytes (HKc) lose contact with the ECM, cells undergo a type of apoptosis termed anoikis. Apoptotic cell death generates modified proteins that induce inflammatory responses. S100 proteins are suggested as HKc-derived mediators of inflammation. In psoriasis, S100A8/S100A9 expression is markedly elevated and aberrant expression is detected in the epidermal layers. We examine how inflammatory agents such as okadaic acid (OA), a phosphatase inhibitor, influence the distribution and promote formation of vesicles containing S100A8/S100A9 in HKc. We propose that formation of these membrane structures involve cytoskeletal proteins, such as tubulin and actin. Our studies show that S100A8/S100A9 assume a cytoplasmic distribution in resting cells. Instead of linear actin bundles typical for HKc, circular arrays of actin bundles formed in the cortical cytoplasm of OA-treated HKc. OA treatment causes S100A8/S100A9 movement to the cell periphery via intact microtubules and localization within the cortical actin ring followed by the formation of S100-positive membrane blebs that protrude from the apical surface. The blebs are ringed by F-actin, which sequesters the S100 protein-containing cytoplasm. OA induces F-actin depolymerization in HKc. Disruption of the F-actin network by OA, as quantified by a decrease in the F/G-actin ratio, does not alter the spatial distribution or formation of the membrane blebs. Bleb integrity is maintained even under increased osmotic force physical stress as measured by FM 1-43 dye incorporation. Cytochalasin D, an F-actin polymerization inhibitor, prevents the formation of blebs observed in OA-treated HKc. Sustained treatment with OA results in apoptosis. The majority of HKc detach from the substrata; only cell debris and intact blebs remain. We propose that S100 protein-containing blebs may then be engulfed by phagocytic cells and invoke the inflammatory response.

1804

**Ezrin Forms a Link Between Signaling and Vesicular Membrane Trafficking in Gastric Acid Secretion**

F. WU,[1] H. Deng,[1] R. Zhou,[2] Z. Guo,[3] Z. Fan,[1] K. Yuan,[1] X. Cao,[1] J. Forte,[2] X. Yao[3]; [1]Division of Cell Dynamics, Hefei National Laboratory, Anhui, China, [2]Molecular & Cell Biology, University of California, Berkeley, CA, [3]Physiology, Morehouse School of Medicine, Atlanta, GA

Stimulation of acid secretion in gastric parietal cells typically involves an initial elevation of intracellular calcium and/or cAMP followed by activation of a cAMP-dependent protein kinase cascade that triggers the translocation and insertion of the proton pump enzyme, H,K-ATPase, into the apical plasma membrane of parietal cells. Our recent studies have revealed PKA-mediated phosphorylation site on ezrin and the importance of such phospho-regulation in gastric acid secretion (J. Biol. Chem. 2003. 278, 35651). However, little is known about the cAMP-dependent signaling events and the role of the actin cytoskeleton in supporting the apical pole remodeling associated with parietal cell activation. Using phospho-mimicking ezrin as bait, we have identified a dozen positive clones including a novel ARF GTPase-activating protein (ARF-GAP). This novel ARF-GAP contains a PH domain and displays a PIP2-dependent GAP activity *in vitro*. The ARF-GAP interacts with ezrin *in vivo* and *in vitro*, and co-distributes with ezrin to the apical membrane of gastric parietal cells. To determine the inter-relationship of ezrin-ARF-GAP localization to the apical membrane, we carried siRNA experiment to suppress ezrin protein level. The apical localization of ARF-GAP is minimized in the absence of ezrin, suggesting the role of ezrin in targeting and/or maintaining ARF-GAP to the apex. Moreover, the translocation of H,K-ATPase to the apical membrane is disrupted in the ezrin-deficient cells. Currently, we are evaluating how ezrin-ARF-GAP interaction orchestrates ARF6 and H,K-ATPase trafficking cycle in parietal cell acid secretion.

1805

**Phosphorylation of Ezrin on T567 is Not Involved in Membrane Recruitment and Activation of Parietal Cells**

R. Zhou, J. Forte; Molecular & Cell Biology, University of California, Berkeley, CA

The ERM protein family (ezrin/radixin/moesin) participates in linking functional activities of the plasma membrane to the actin cytoskeleton, and are regulated via phosphorylation on several potential sites. Phosphorylation of the conserved threonine residue near the C-terminus (T567, ezrin; T558, moesin; T564, radixin) is under the control of the small GTPase Rho, and has been shown to alter N/C oligomerization of ezrin and promote formation of actin-rich surface projections in many cells. In previous work using the gastric parietal cell model for secretory activation we compared wild type T567 ezrin (WT) and mutant forms, including the non-phosphorylatable T567A and one mimicking permanent phosphorylation, T567D. The most striking result was that expression of T567D (in contrast to WT and T567A) produced a great elaboration of cytoplasmic extensions, but principally at the basolateral surface, and these cells did not respond to secretory stimuli. We concluded that T567 phosphorylation may direct ezrin to membrane-cytoskeletal activity away from apical secretory surface. Here we show that the elaborate basolateral actin- and ezrin-rich extensions induced by T567D mutant ezrin "parasitizes" many membrane sources for the requisite increase in area that must occur, including H pump-rich tubulovesicles and even apical membrane. Using a site-specific antibody we show that a fraction of total ezrin is phosphorylated at T567 in resting, non-stimulated, parietal cells, and that there is no significant change in the level of T567 ezrin (or T558 moesin) when the cells are maximally stimulated to secrete acid. These data support the hypothesis that the Rho signaling pathway and T567 phosphorylation are not involved in membrane recruitment and secretory activation at the apical plasma membrane of the parietal cell, but probably function to support membrane-cytoskeletal interaction at the basolateral surface.

1806

**Missense Mutations in the Merlin Protein Cause a Loss of Association with Lipid Rafts**

J. L. Ovesen, J. Stickney, L. Foster, W. Ip; Cell Biology, University Of Cincinnati, Cincinnati, OH

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder affecting 1:40,000 live births. NF2 results from loss of heterozygosity at the *NF2* locus and the accompanying loss of functional gene product, the canonical tumor suppressor merlin. Loss of functional merlin leads to formation of schwanommas of the 8$^{th}$ cranial nerve and other nervous system tumors. Merlin is related to the ERM (ezrin-radixin-moesin) family of cytoskeleton-membrane linker proteins. Despite the fact that merlin lacks the C-terminal F-actin binding site that is conserved in the ERM proteins it is insoluble in non-ionic detergents such as Triton X-100 (TX-100), a characteristic often attributed to cytoskeleton-associated proteins. However, our recent observation that merlin resides in lipid rafts, Triton-insoluble membrane microdomains rich in cholesterol and sphingolipids, led us to hypothesize that the detergent solubility properties of merlin result mainly from its localization to rafts with limited contribution from binding to the F-actin network. We tested this hypothesis by investigating the TX-100 solubility and raft localization of *NF2* missense mutations K79E, L360P, and L535P. As shown previously, all of these mutations encode merlin proteins with greater detergent solubility than wild type merlin. Biochemical and immunofluorescence studies further revealed that all three mutations cause a dramatic loss of lipid raft association. Future studies will probe whether re-targeting of these mutations to rafts using lipid anchors can restore their growth-suppressive function and will test our central hypothesis that lipid raft localization of merlin is necessary for its function. Supported by the NIH, the NNFF, and the DOD.

1807

**Filamin-Caveolin Interactions in Caveolae-Mediated Endocytosis**

M. Sverdlov, R. D. Minshall; Pharmacology, University of Illinois at Chicago, Chicago, IL

The F-actin cross-linking protein filamin A also binds to caveolin-1, the primary coat protein of caveolae (Stahlhut and van Deurs, MBC 11:325-337, 2000). This interaction is thought to mediate the physical association between caveolae and actin filaments. In the present study, we tested the functional importance of filamin A in caveolae-mediated endocytosis. Filamin A co-fractionated with caveolin-1 in lipid raft-enriched fractions of human lung microvascular endothelial cells. In addition, caveolin-1 co-immunoprecipitated with filamin A suggesting that these proteins are associated *in vivo*. Expression of a filamin A fragment containing the caveolin-1 binding site (repeats 22-24) prevented the association of caveolin-1 with endogenous filamin A and significantly reduced albumin endocytosis. We next determined if caveolin-filamin interactions are regulated by caveolin-1 phosphorylation on Tyr 14, which is *Src*-dependent. mSIRK, an activator of Gβγ, which in turn induces *Src* activation (Shajahan et al., JBC 279:48055-48062, 2004), or sodium orthovanadate, an inhibitor of tyrosine phosphatases, induced caveolin-1 phosphorylation and its association with filamin. The increase in caveolin-filamin binding was blocked by PP2, a specific inhibitor of *Src*-family tyrosine kinases. Furthermore, we found that filamin A associates to a greater extent with the phosphorylation-mimicking caveolin-1 mutant, Y14D, compared to the phosphorylation-defective caveolin-1 mutant, Y14F. Thus, our results indicate that caveolin-1 phosphorylation positively regulates the interaction between filamin A and caveolin-1. This interaction is now shown to be required for *Src*-dependent, caveolae-mediated endocytosis in endothelial cells.

## Extracellular Matrix & Cell Behavior I (1808-1823)

1808

**Laminin Isoforms Regulate Trophoblast Cell Behavior During Mouse Embryo Implantation**

E. J. Klaffky,[1] I. M. Gonzales,[1] J. Miner,[2] F. J. De Mayo,[3] J. P. Lydon,[3] A. E. Sutherland[1]; [1]Cell Biology, University of Virginia, Charlottesville, VA, [2]Internal Medicine, Washington University, St. Louis, MO, [3]Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX

Extracellular matrix has specific effects on cell behavior which influence many aspects of early development. In the early postimplantation mouse embryo dynamic patterns of laminin (Ln) 1in the embryo and Ln 10/11 expression in the surrounding decidua correlate with the progression of implantation. These two Ln isoforms have distinct effects on trophoblast cell behavior : Ln 1 promotes random migration and decreases spreading, whereas Ln 10/11 promotes both spreading and persistent migration. When presented as adjacent substrates however, cells recognize the boundary and do not enter the region containing Ln 1. Lns 1 and 10/11 also have differential effects on cell-cell adhesion, with Ln 1 decreasing and Ln 10/11 promoting intercellular adhesion. Trophoblast cells interact with Ln 1 through beta 1 integrins, whereas they interact with Ln 10/11 with a combination of beta 1 and beta 3 integrins. The effects of each Ln isoform on cell behavior and the localization of Ln 1 in Reichert's membrane and Ln 10/11 in the surrounding decidual matrix suggest that they may influence the progress of implantation. To test this possibility we generated a decidual knockout of Ln 10/11 by crossing a floxed Ln alpha 5 mouse with one carrying Cre under the control of the progesterone receptor promoter. Females homozygous for the conditional locus and carrying the Cre gene are fertile, although the decidual matrix completely lacks Ln 10/11. Embryos are able to initiate implantation, however, the pattern of trophoblast invasion and morphogenesis of the yolk sac placenta are abnormal. These results suggest that the spatial distribution of Ln isoforms is a key factor regulating the direction and quality of invasion of trophoblast cells during implantation, and that decidual Ln 10/11 provides epigenetic cues that drive the morphogenesis of the yolk sac placenta.

1809

**Detection of Tenascin-W in Patient Sera and in Cancer Tissue**

M. Degen,[1] C. Rüegg,[2] P. Schraml,[3] R. Simon,[3] R. Kain,[4] R. Chiquet-Ehrismann[1]; [1]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland, [2]Centre Pluridisciplinaire d'Oncologie, University of Lausanne, Lausanne, Switzerland, [3]Institute of Pathology, University of Basel, Basel, Switzerland, [4]Department of Ultrastructural Pathology and Cell Biology, University of Vienna, Vienna, Austria

It is well recognized that extracellular matrix proteins such as tenascins are present during the development of an organism as wells as in pathological situations. However, the functional impact of their presence is not very well understood so far. Tenascin-W (TN-W), the fourth member of the tenascin family remains the least well characterized one. In our study we aim to determine serum TN-W levels in cancer patients evaluating its diagnostic significance, and to investigate its expression in tumors. To measure serum TN-W levels, a sensitive sandwich ELISA was established by the use of two anti-human TN-W antibodies and the purified protein as a standard. With our assay, concentrations as low as 0.005 mg/l are detectable, making it sufficiently sensitive for the determination of serum TN-W levels. TN-W concentrations in sera of patients with

breast cancer were not increased compared to healthy volunteers (normal: 0.424 +/- 0.13 mg/l; breast cancer: 0.370 +/- 0.173 mg/l). However, TN-W concentrations in colon cancer patients were elevated (colon cancer: 0.904 +/- 0.34 mg/l). Furthermore, we analyzed TN-W expression in human breast tumor extracts by western blotting. Interestingly, we found TN-W expression in tumor tissues (carcinomas as well as benign tumors), but no expression in the normal mammary parenchyma. 74% of the tumor samples were TN-W positive and 82% showed expression of TN-C. However, TN-W and TN-C amounts vary greatly between tumors and some contain either TN-W or TN-C exclusively, indicating independent mechanisms regulating TN-W and TN-C expression. We conclude that TN-W might be an important protein with diagnostic value in cancer patients.

1810

**Multivalent Action of NG2 Proteoglycan in Cell-Microenvironment Interactions Involved in Tumor Growth and Metastasis**

S. Cattaruzza,[1,2] E. De Lorenzo,[1] W. Stallcup,[3] A. Colombatti,[1] P. Braghetta,[4] P. Bonaldo,[4] R. Perris[1,2]; [1]Division for Experimental Oncology 2, The National Cancer Institute CRO IRCCS, Aviano (PN), Italy, [2]Department Evolutionary and Functional Biology, University of Parma, Parma, Italy, [3]The Burnham Institute, LaJolla, CA, [4]Department of Medical Biotechnology, University of Padua, Padua, Italy

Using cells derived from knock-out mice and a combined RNAi and gene transfer approach in human mesenchymal tumor cells we find that NG2 proteoglycan is critically involved in the cells' autocrine and paracrine response to several FGF family members. The proteoglycan contributes to the control of FGF signalling by specifically associating, in a ligand-independent manner, with FGFR1/3 and by sequestering and activating FGFs on the cell surface. Contemporary, we also confirm that NG2 serves as a integrin-independent receptor for collagen type VI and that it promotes tumor cell motility on this substrate through the activation of cytoskeleton-mediated signal transduction cascades. A phospho-proteomic approach has been adopted to dissect these pathways and determine the extention to which they converge with those elicited by growth factors. To further clarify the transcriptional regulation underlying this phenomenon, we are pursuing a global gene profiling by DNA microarray in cells overexpressing NG2. Similar NG2-rich melanoma cells, sarcoma cells in which the constitutive PG levels have been knocked-down by RNAi, and dominant-negative mutant cells are examined in motility assays in vitro and for their ability to grow and metastasise in wild type and collagen type VI knock-out animals. These studies are complemented by trans-endothelial migration assays in vitro, involving primary haematic and lymphatic cells, which are aimed at defining the molecular mechanisms responsible for the ability of NG2 to confer metastatic potential to transformed cells by affecting their intra- and extravasation capacities.

1811

**β1,4-*N*-Acetylglucosaminyltransferase III Antagonizes the Effect of β1,6-*N*-Acetylglucosaminyltransferase V on α3β1 Integrin-mediated Cell Migration**

Y. Zhao,[1] J. Gu,[1] T. Nakagawa,[2] E. Miyoshi,[1] Y. Kariya,[3] K. Miyazaki,[3] A. Kondo,[2] N. Taniguchi[1]; [1]Department of Biochemistry, Osaka University Graduate School of Medicine, Osaka, Japan, [2]Department of Glycotherapeutics, Osaka University Graduate School of Medicine, Osaka, Japan, [3]Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan

β1,6-*N*-acetylglucosaminyltransferase V (GnT-V) is required in the biosynthesis of β1,6GlcNAc-branched *N*-linked glycans attached to cell surface and secreted glycoproteins. Malignant transformation is accompanied by increased β1,6GlcNAc-branching of *N*-glycans. β1,4-*N*-acetylglucosaminyltransferase III(GnT-III) catalyzes the formation of a bisecting GlcNAc structure in *N*-glycans, resulting in the suppression of further processing and the elongation of *N*-glycans catalyzed by GnT-V in vitro, since it is not able to use the bisected oligosaccharide as its substrate. It has long been hypothesized that the suppression of β1,6GlcNAc branching by the inhibition of GnT-V would be possible based on substrate specificity studies. To test this, we drew a comparison among MKN45 cells(a human gastric cancer cell line), which were transfected with GnT-III, GnT-V, or both, respectively. We found that α3β1 integrin-mediated cell migration on Laminin5 was greatly enhanced in GnT-V transfectant. Such enhancement of cell migration was significantly blocked after introduction of GnT-III. Interestingly an increase in the bisected GlcNAc but a decrease in β1,6 GlcNAc branched *N*-glycans on α3 integrin was observed in these double transfectants. Consistent with that, the overexpression of GnT-III inactive mutant failed to induce all changes above. On the other hand, the introduction of GnT-III had no effects on both GnT-V'activities and expression level of α3 integrin. Our results clearly demonstrate for the first time that GnT-III and GnT-V can modify the same target glycoprotein positively and negatively respectively, by regulating sugar chains on it and the priority of GnT-III for modification of α3 integrin maybe one of the reasons why GnT-III inhibits GnT-V induced cell migration.

1812

**Beclin/ATG6 Regulates Proliferation and Survival During 3D Morphogenesis and Anoikis**

J. Debnath,[1] G. Gao[2]; [1]Department of Pathology, University of California San Francisco, San Francisco, CA, [2]Department of Cell Biology, Harvard Medical School, Boston, MA

The individual units (acini) comprising glandular epithelium possess a hollow lumen that is filled during early oncogenesis. We have been investigating the generation of luminal space in epithelial acini using an *in vitro* 3D MCF-10A cell culture model. Lumen formation involves the selective apoptosis of centrally-located cells within developing acini. However, the hollow lumen still forms when classical apoptosis is blocked via Bcl-2 overexpression, indicating additional processes contribute to luminal clearance. During lumen formation, the central cells in both normal and Bcl-2 expressing acini contain numerous autophagic vacuoles, suggesting autophagy, an adaptive catabolic process in which a cell digests its own contents, may influence cell fate in the lumen. To elucidate the role of autophagy in epithelial cells, we are examining how autophagy regulatory molecules (called ATGs) influence 3D morphogenesis. Remarkably, RNAi-mediated downregulation of Beclin/ATG6 (a tumor suppressor) enhances proliferation in developing acini. Although the resulting acini grow larger in size, they exhibit luminal apoptosis and remain hollow. Surprisingly, Beclin/ATG6 knockdown does not cooperate with Bcl-2 mediated apoptotic inhibition to fill the lumen, despite the decreased autophagy and increased proliferation in these structures. Because cells in the lumen lack contact with extracellular matrix, we have further investigated how autophagy influences cell death upon matrix detachment (*anoikis*). Matrix detachment strongly induces autophagy, confirmed by electron microscopy and the relocation of light-chain 3 (LC3/ATG8) to autophagosomes. Importantly, matrix-detached cells still exhibit autophagy when apoptosis is blocked by Bcl-2 overexpression. In contrast, Beclin/ATG6 knockdown inhibits autophagy and attenuates the cytoprotective effects of Bcl-2 during *anoikis*. Hence, autophagy may actually promote cell survival during matrix detachment. We are now investigating if these prosurvival functions of Beclin/ATG6 influence luminal cell fate and how other ATGs regulate epithelial proliferation and survival during 3D

morphogenesis and *anoikis*.

1813

**Gene Expression Patterns in Aging Human Discs Show Quiescent and Transcriptionally Active Cells**

H. E. Gruber, J. A. Ingram, E. N. Hanley; Orthopaedic Surgery, Carolinas Medical Center, Charlotte, NC

Gene expression patterns in aging human discs show quiescent and transcriptionally active cells Disc cell functions include appropriate gene expression of tissue-specific extracellular matrix (ECM), ECM remodeling, and cell maintenance. Aging/degeneration introduce radical changes in disc physiology. Our objective was to assess global gene expression (using gene arrays) and cellular gene expression (using in situ hybridization) in healthy and degenerating discs in Human Subjects Institutional Review Board-approved protocols. Human disc tissue, Thompson grades II-IV, was obtained from surgeries or control donors. Laser capture microdissection harvested cells for microarray analysis to obtain global expression profiles (9 lumbar, 2 cervical specimens; 4 grade II discs, mean age 34.7 yrs; 4 grade III discs, mean age 41.5 yrs, and 3 grade IV discs, mean age 62.3 yrs) using Affymetrix arrays. In situ hybridization identified expression of selected genes at the cellular level. Strong expression signals were found for collagen type I alpha 2, pro-alpha I type II collagen, fibronectin 1, SPARC, aggrecan, SOX9, MMP3, TIMP3, chondroitin-6-keratan sulfotransferase 1, latent TGF-ß binding protein 2ß, IGF binding protein 7, pro-apoptotic caspases 3 and 8, and anti-apoptotic BCL-2 beta protein. SPARC expression correlated with age ($p=0.04$, $r^2= 0.451$). Pro-alpha 1 collagen II gene expression correlated with age ($p=0.04$, $r^2= 0.447$). Unexpectedly, in situ hybridization analysis revealed that not all cells were transcriptionally active for a given gene product. Inability of adjacent cells to coordinate gene expression is an important finding which would have been missed with only global gene expression profiling. Possible explanations are that with aging/degeneration: a) cells become fewer in number and loose ability to coordinate gene expression, b) loss of signals/cytokines requisite for coordinated gene expression, c) quiescent cells are at different levels of differentiation or senescence.

1814

**Alterations and Functional Consequences of Changes in Laminin Composition of the Extracellular Matrix Induced by the Senescence Program in Prostate Epithelium**

C. C. Sprenger; Medicine, University of Washington, Seattle, WA

The microenvironment in which cells reside appears to play a role in determining behavior of those cells. Several studies support the importance of a changing microenvironment in both aging of an organism as well as in the progression of various cancers. The ability of cells to stop replicating but remain metabolically active is termed senescence. In young organisms, senescence may prevent epithelial cancers by stopping replication of damaged cells, but increase the incident of such cancers in old organisms, in part, by altering the tissue microenvironment. Currently, only senescent epithelial cells have been detected in human prostate. Yet, the role senescent epithelial cells play in the alteration of the microenvironment and prostate cancer progression remains to be elucidated. We hypothesized that as part of the senescent program in prostate epithelial cells, a change in the laminin component of the ECM occurred, which, in part, altered the microenvironment such that growth and migration of the epithelial cells was altered. Senescent prostate epithelial cells express high levels of mac25; overexpressing this protein in the metastatic M12 prostate cancer cell line led to cell senescence, a delay in G1, and decreased growth/tumorigenicity in vivo. We demonstrated a shift in laminin production from laminin 5 ($\alpha3, \beta3, \gamma2$) and some laminin 8 ($\alpha4, \beta1, \gamma1$) in the M12 cells to laminin 9 ($\alpha4, \beta2, \gamma1$) in the M12mac25 cells. Further, matrix from M12mac25 cells reduced the migratory and invasive behavior of the M12 cells in vitro. In conclusion, senescence in prostate epithelial cells induced by the senescence-associated gene mac25 altered the laminin composition of the matrix and this M12mac25 matrix reduced migration and invasion of the metastatic M12 prostate cancer cell line.

1815

**Histological Studies Demonstrating the Effect of a Collagen-Like Peptide on ECM Molecules Synthesis in Human Skin**

C. Dal Farra, A. Perrin, E. Bauza, N. Domloge; Vincience, Sophia antipolis, France

Recent studies have shown that, in addition to improving *ex vivo* skin morphology, the newly developed synthetic oligo collagen-like peptide enhances cultured cell adhesion and differentiation. In these studies, we carried out histological analysis of the effects of a collagen-like peptide and compared it with vitamin C, which is known for its ability to enhance the synthesis of ECM molecules such as collagen. We performed time course studies and immunostaining analysis on human *ex vivo* skin samples treated with 1% collagen-like peptide for 16h to 72h. Parallel experiments with vitamin C at 20 µg/ml were also conducted. Immunostaining analysis included evaluations of collagen I, III, IV, laminin 5, beta 1 integrin, filaggrin and pan keratin. Our findings revealed that application of collagen-like peptide enhanced the synthesis of these ECM molecules, and this effect was seen as early as 16h after application for some molecules such as laminin 5, collagen III, IV and pan keratin. The expression of the other molecules increased after different periods of time of application of the peptide. Interestingly, the synthesis response of some ECM molecules, such as laminin 5, collagen III, IV and pan keratin, was greater and more rapid when prompted by collagen-like peptide than by vitamin C. The findings also revealed that both active ingredients stimulated ECM molecules synthesis to a similar degree when evaluations were made after a longer period of time. A few proteins, such as collagen IV and beta 1 integrin maintained a superior synthesis after a longer period of time in response to the peptide. Within the context of anti-aging and photoaging skin care products, these histological studies reveal that collagen-like peptide's stimulating effect on ECM molecule synthesis can be of great use.

1816

**Substrate Geometry and Chemistry Co-Regulate Growth and Differentiation of Nerve, Schwann, and Muscle Cells**

O. Kolotushkina,[1] M. R. Hynd,[2] W. Shain,[2] B. L. Patton[1]; [1]CROET, Oregon Health & Science Univ., Portland, OR, [2]Wadsworth Center, New York Department of Health, Albany, NY

Genetic studies have confirmed an array of extracellular matrix proteins influence growth and differentiation of muscle cells, Schwann cells, and axons in the developing neuromuscular system. Matrix proteins have restricted distributions in endodneurial and muscle basal laminae, and are thought to have synergistic effects on cellular differentiation. However, in vitro assays have been limited to uniform culture substrates and micropatterning of single components. To begin to assess information encoded in the spatial complexity of multicomponent substrates, we adapted microcontact printing methods to derivitized polyacrylamide "hydrogel" substrates. Matrix proteins double-labeled with fluorescent chromophore

and biotin were stamped at non-saturating levels on streptavidin-conjugated hydrogels. Complexity was increased by serial deposition of multiple proteins, in distinct patterns. Cells (hippocampal neurons; C2C12 myoblasts; primary Schwann cells) adhered poorly (neurons) or not at all to bare hydrogel. Thus, behaviors resulted predominantly from interactions with bound proteins and soluble factors. Schwann and muscle cells proliferate at high rates and move apart from each other on uniform laminin-1 substrates. Cell proliferation was significantly reduced on linear (10 micron-wide) substrate geometries, and cells became aligned, similar to arrangements in vivo. The results suggest availability of embryonic matrix surfaces may limit proliferation, and promote myofiber alignment. Neurons extend processes without orientation on unpatterned substrates. In contrast, neurons on crossed linear patterns of polylysine and laminin-2 appeared to preferentially form axons on laminin-2, and dendrites on polylysine. Axonal preferences were not observed on laminin-1/polylysine patterns. The results suggest that matrix chemistry and geometry have independent and co-dependent roles in regulating tissue formation, possibly including orientation of neuronal polarity. Nerves and muscles regenerate imperfectly following injury in adults. Combinatorial micropatterned substrates may increase the efficacy of cellular guides in tissue engineering. *Supported by Nanobiotechnology Center (NBTC), NSF*.

1817

**Vitronectin is Essential for the Differentiation of Cultured Mouse Cerebellar Granule Cell Precursors**

Y. Miyamoto,[1] T. Saitou,[1] H. Ambe,[1] M. Takeda,[1] S. Murase[2]; [1]Department of Biology, Ochanomizu University, Tokyo, Japan, [2]Department of Cell Biology, Univiersity of Virginia, Charlottesville, VA

Proliferation and neuronal differentiation of granule cell precursors (GCPs) are regulated spatially and temporally in the postnatal cerebellum. The proliferating activity of GCPs is regulated by the Sonic hedgehog protein (Shh), however, it is not fully understood why the GCPs stop proliferation and begin to extend their neurites at the molecular layer in the cerebellum. It has been reported that an extracellular matrix protein, vitronectin, is important for the cerebellar morphogenesis in chick and rat, and one of the vitronectin receptors, αvβ5 integrin heterodimer, plays a critical role for the neurite elongation. Our recent results showed that vitronectin arrests the Shh-dependent proliferation of GCPs, and suggested that one of the immunoglobulin superfamily proteins, nectin-like protein-5 (Necl-5), plays a role in the differentiation of GCPs. In this study, we examined the effect of vitronectin or Necl-5 on the differentiation of mouse GCPs using primary culture system. First, we observed the increased expression level of β5 integrin subunit in the differentiating GCPs. To examine the effect of vitronectin or Necl-5 on the β5 expression, GCP cells was plated on vitronectin-coated dishes or cultured with the existence of anti-Necl-5 function blocking antibody. Obtained results showed that vitronectin increased the expression level of β5 subunit and anti-Necl-5 antibody suppressed its expression. Finally, to confirm the function of vitronectin for the GCPs differentiation, GCPs from vitronectin knockout mice were cultured with or without exogenous vitronectin. The vitronectin$^{-/-}$ GCPs did not differentiate into the mature granule cells, and the extent of their neurite elongation was smaller than that of wild-type GCPs. These defects were recovered by culturing the vitronectin$^{-/-}$ GCPs on vitronectin-coated dishes. These results demonstrate that vitronectin is essential for the differentiation of cultured GCPs.

1818

**Differential Sulfation Pattern of Chondroitin Regulates Axonal Guidance**

Y. Katagiri, H. Wang, H. M. Geller; NHLBI, NIH, Bethesda, MD

Failure of injured axon to regenerate within the CNS after injury has been attributed to inhibitory properties of the glia scar. Others and we showed the presence of chondroitin sulfate proteoglycans (CSPG) forms a barrier to axon outgrowth. Recent studies in vivo demonstrated that removal of CSPG glycosaminoglycan (GAG) chains by chondroitinase ABC treatment promotes functional recovery after injury, indicating GAG chains are essential for the activity. To evaluate functional roles of GAG chains, we have used immobilized GAG chains as well as CSPG to form an interface on poly-L-lysine coated glass coverslips and checked whether axons can cross its boundary. We found that CS-A GAG chains, mainly consisting of 4-sulfated GalNAc in the disaccharide unit, repelled axonal outgrowth of mouse cerebellar granule neurons. In contrast, CS-C consisting of 6-sulfated GalNac, showed no activity. Treatment of GAG chains with 4- sulfatases abolished their inhibitory effect. These results are consistent with the fact showing that inhibition of sulfation in cultured astrocytes by several drugs reduced the inhibitory activity in glia scar model. Further, exposure to CS-A as well as CSPG, but not CS-C, induced RhoA activation in PC12 cells. This finding revealed that the sulfation pattern of chondroitin is pivotal for its inhibitory activity and downstream signal transduction and demonstrates the importance of 4-sulfation on CSPG in axon regeneration.

1819

**Human-, Mouse, and Rat-Laminin-5 May Have Differing Effects on Oral Squamous Cell Carcinoma Proliferation *In Vitro***

T. Vinh, S. O'Malley, K. Kingsley; Biomedical Sciences, University of Nevada, Las Vegas School of Dental Medicine, Las Vegas, NV

The majority of oral cavity cancers are squamous cell carcinomas. Even though they represent only about 3% of all cancers in the United States, they are disproportionately important because half result in death within five years. Because of their asymptomatic progression, they have usually metastasized by the time the primary lesion is discovered. Therefore, detection of oral squamous cell carcinoma (OSCC) before metastasis is critical for a hopeful diagnosis. Laminin-5 is a key extracellular matrix (ECM) protein that is preferentially produced by OSCC, and cleaved by matrix metalloproteinases (MMPs) secreted by OSCC in order to metastasize through the ECM. This study examines the extent to which Laminin-5 affects OSCC behaviors, such as proliferation, *in vitro*. Collagen Type I and Fibronectin were used as positive controls, and poly-L-lysine and untreated plastic as negative controls, all of which were used in OSCC 0, 1, 2, and 3-day proliferation assays. Current literature surveys reveal experimentation using mouse and rat Laminin-5 with human OSCC despite significant differences between their amino acid sequences. Our results suggest that human Laminin-5 positively influences OSCC proliferation *in vitro* significantly over mouse Laminin-5. This research is part of the foundation for our understanding of OSCC behaviors, such as proliferation, and the subsequent metastasis in oral cancers.

1820

**The Globular Domains of Human and Rat Laminin-5 Alpha3,Beta3, and Gamma2 Subunits May Contain Distinct Alpha3Beta1 Integrin Binding Sites that Influence *In Vitro* Adhesion of Oral Squamous Cell Carcinoma Cells**

G. Koontz, K. Kingsley; Biomedical Sciences, University of Nevada, Las Vegas School of Dental Medicine, Las Vegas, NV

Laminin-5 is an extracellular matrix glycoprotein involved in modulating cellular behaviors, such as adhesion and migration. Many studies currently use rat-derived laminin-5 to examine human cell adhesion, tissue remodeling, and migration *in vitro*. Our study objective is to understand the structural and functional relationships between alpha3 beta1 and alpha6 beta4 integrins that modulate adhesion to, and migration on, laminin-5 and different laminin-5 sources through a comprehensive analysis of the sequence similarities and differences between the human- and rat-derived laminin-5 subunits. Using BLAST2 analysis software, we show the primary amino acid sequences from the National Center for Biotechnology Information (NCBI) of human and rat laminin-5 alpha3-subunits contain specific, homologous alpha3 beta1 integrin-binding domains. We also report distinct alpha3 beta1 integrin-binding domains not shared between rat and human alpha3-subunits of laminin-5. In addition, using ProtScale and MultiCoil software, differences in coiled-coil formation and hydrophobicity between the globular domains of these subunits were found. We conclude these domains may be capable of supporting integrin ligation and may therefore represent functional differences between rat and human alpha3-subunits of laminin-5 that modulate specific cellular responses, such as adhesion and migration.

1821

**Biochemical Cleavage of Laminin-5 by Matrix Metalloproteinases May Alter Specific Integrin Binding Sites on Specific Laminin Subunits**

C. L. Neilson, S. O'Malley, K. Kingsley; Biomedical Sciences, University of Nevada, Las Vegas School of Dental Medicine, Las Vegas, NV

Laminin-5 is an extracellular matrix glycoprotein involved in modulating cellular behaviors, such as adhesion and migration. Many studies demonstrate that specific laminin-5 subunits, such as gamma-2 are cleaved by matrix metalloproteinases (MMPs), such as MT1-MMP. Our study objectives are (1) to understand the relationship between the release of specific matrix metalloproteinases from Oral Squamous Cell Carcinomas (OSCC), (2) the specific cleavage sites of laminin-5 subunits by these matrix metalloproteinases and (3) the relationship of this OSCC-mediated MMP-cleavage to OSCC adhesion *in vitro*. We hypothesize that primary amino acid sequences from the National Center for Biotechnology Information (NCBI) of laminin-5 subunits contain specific sequences that are known cleavage sites of MMPs. Using BLAST2 analysis software, we report distinct enzymatic cleavage sites that may be responsible for the altered phenotypes between normal and proteolyzed laminin-5 that modulate specific cellular responses, such as adhesion and possibly cell migration in OSCC.

1822

**The Laminin Derived Peptide YIGSR Modulates Adhesion Molecules in an Adenoid Cystic Carcinoma Cell Line**

R. Jaeger,[1] E. Oliveira,[1] C. Furuse,[2] V. Araujo,[2] V. Freitas[1]; [1]Cell and Developmental Biology, ICB-USP, Sao Paulo, Brazil, [2]Sao Leopoldo Mandic Research Institute, Sao Paulo, Brazil

Adenoid cystic carcinoma is a malignant salivary gland neoplasm with high level of recurrence and distant metastasis. We have demonstrated that laminin-1 modulates the phenotype of a cell line (CAC2) derived from human adenoid cystic carcinoma. We are currently studying the expression of a non-integrin laminin receptor, the 32/67kDa laminin-binding protein (LBP), in adenoid cystic carcinoma. Immunohistochemistry showed the presence of LBP in adenoid cystic carcinoma cells in vivo and in vitro. The LBP binds a sequence of laminin beta 1 chain, the YIGSR peptide. This peptide promotes cell attachment and migration. We decided to study the role played by the peptide YIGSR and its ligand, the LBP receptor, in CAC2 cells. These cells were grown inside either laminin-1 (control) or laminin-1 enriched with YIGSR (treated). Cells treated by YIGSR appeared non-cohesive, with fibroblastic-like morphology, while control cells were epithelioid and closely packed. This result suggested decrease of adhesion molecules induced by YIGSR. Thus, we decided to study the role played by the peptide YIGSR, and the LBP receptor, on the expression of adhesion molecules in CAC2 cells. Samples were analyzed by immunoblot to detect E-cadherin and beta-catenin. YIGSR decreased the amount of these proteins in CAC2 cells, when compared to controls. Pretreatment of cells with anti-LBP restored adhesion molecules expression. The result was confirmed by whole mount immunofluorescence. Cells grown inside laminin-1 exhibited E-cadherin and beta-catenin as a linear staining located at cell boundaries. Cells treated by YIGSR were non-cohesive, with a discrete punctate label. Blockage of LBP restored the linear pattern of E-cadherin and beta-catenin. Our results suggested that YIGSR, and its ligand the 32/67kDa LBP regulate the expression of adhesion molecules in an human adenoid cystic carcinoma cell line

1823

**Cleavage of Laminin-5 by Matrix Metalloproteinase-2 May Alter Specific Integrin Binding Sites on Laminin Subunits**

L. A. Trerice, S. O'Malley, K. Kingsley; Biomedical Sciences, University of Nevada, Las Vegas School of Dental Medicine, Las Vegas, NV

Laminin-5 is an extracellular matrix glycoprotein involved in modulating cellular behaviors, such as adhesion and migration. Previous research has shown that laminin-5 subunits, such as gamma-2 are cleaved by specific matrix metalloproteinases (MMPs), such as MMP-2. Our study objectives are (1) to identify MMPs that are capable of performing enzymatic cleavage of laminin-5 subunits and (2) identifying the specific cleavage sites of the laminin-5 subunits that may be acted upon by these MMPs and (3) characterizing the enzymatic cleavage and the effect on cellular adhesion *in vitro*. We hypothesize that primary amino acid sequences from the National Center for Biotechnology Information (NCBI) of laminin-5 subunits contain specific sequences that are known cleavage sites of MMPs. Using BLAST2 analysis software, we propose alternative enzymatic cleavage sites that may be responsible for the observed altered phenotypes between normal and proteolyzed laminin-5 that modulate specific cellular responses, such as adhesion and possibly cell migration in OSCC.

## Extracellular Matrix & Morphogenesis (1824-1839)

1824

**Human Mesenchymal Stem Cells Enhance Angiogenesis within Denser 3D Fibrin Matrices via Initial MMP-9 and pro-MMP-2 Upregulation**

C. M. Ghajar,[1] S. C. George,[1] A. J. Putnam[2]; [1]Biomedical Engineering, University of California, Irvine, Irvine, CA, [2]Biomedical Engineering and Chemical Engineering and Materials Science, University of California, Irvine, Irvine, CA

The diffusion limitation is the most prominent obstacle to developing thick, engineered tissues. To overcome this hurdle, our lab has developed a 3D fibrin-based extracellular matrix in which embedded human umbilical vein endothelial cells rapidly form a vascular-like network when cultured in optimal conditions. One limitation of this system is that the fibrin matrix lacks the mechanical integrity necessary for its intended application.

The purpose of this project is to understand how enhancing the mechanical properties of the fibrin gel affect the angiogenic process. Fibrin stiffness was modified by varying the initial concentration of fibrinogen from 2.5-10.0 mg/ml. Compressive moduli were obtained for these conditions, and accompanying changes in the network structure were visualized via confocal microscopy by utilizing Oregon Green-488 conjugated fibrinogen. For each condition, total capillary length was quantified at separate time points (days 7, 14, and 21). The resulting data demonstrates that increasing matrix stiffness significantly decreases the total length of these networks. Adding an exogenous inhibitor (GM6001) of secreted matrix metalloproteinases (MMPs; specifically MMP-2 and -9) to the low stiffness culture duplicated the effect of increased matrix stiffness on total network length. This led to the hypothesis that enhancing the expression of these MMPs within stiffer matrices would abrogate the effect of matrix stiffness on capillary morphogenesis. Adding human mesenchymal stem cells throughout denser fibrin matrices achieved this end (as assayed via gelatin zymography) and led to a significant increase in sprouting by day 21. The notion that this result is MMP-dependent was validated by adding GM6001 to these cultures and observing a subsequent decrease in total network formation. These results delve into the biological processes and engineering design principles that regulate vascularization of tissues in three dimensions.

1825

**Fibroblast Growth Factor (FGF) Signaling Regulates the Expression and the Activity of Matrix Metalloproteases (MMPs) during Mouse Submandibular Gland (SMG) Branching Morphogenesis**

I. T. Rebustini, M. P. Hoffman; MMU-CDBRB, NIDCR-NIH-DHHS, Bethesda, MD

SMGs develop by branching morphogenesis that involves extracellular matrix (ECM) remodeling. FGF signaling plays a key role in ECM remodeling by regulating the expression of ECM proteins and proteases such as MMPs. However, the roles of specific MMPs during SMG morphogenesis are not well understood, therefore we have investigated how FGF signaling regulates the expression and the activity of MMPs. We use embryonic, post-natal and adult mouse SMG to generate developmental profiles of gene expression by real-time PCR, and we found that soluble MMP2 and 9, transmembrane MMP14, 15 and 16, ADAM (A Desintegrin and MMP) 8, 9, 10, 12, 15, 17 and 19, and TIMP (Tissue Inhibitor of MMP) 1, 2 and 3 increase their expression during the early stages of SMG development. Blocking MMP activity using the broad spectrum MMP-inhibitor GM6001 decreases branching morphogenesis in SMG organ cultures. The knockdown of FGFRs by siRNAs downregulates MMP2 expression and activity, suggesting that MMP2 is regulated by FGF signaling. The knockdown of multiple MMPs is required to decrease branching morphogenesis in organ cultures. FGF7 and FGF10 induce distinct epithelial morphologies therefore we analyzed the changes on the FGFRs and MMPs expression in SMG epithelial cultures. FGFRs increase after 6h with FGF7 or FGF10 treatments, whereas MMP2, 14 and 16 peak after 18 hours. MMP2 and MMP16 show similar expression profiles in FGF7-epithelial cultures indicating that they are closely regulated. Also, siRNA knockdown of FGFRs in epithelial cultures downregulates MMP2. In conclusion, FGF signaling regulates the expression and the activity of MMPs during SMG branching morphogenesis.

1826

**Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) Regulates Mammary Gland Development Through Synergy Between Two Substrates, Collagen Type I and Matrix Metalloproteinase-2**

M. Egeblad,[1] B. S. Wiseman,[1] M. D. Sternlicht,[1] H. Kouros-Mehr,[1] K. A. Green,[2] H. Mananzan,[1] K. Holmbeck,[3] H. Birkedal-Hansen,[3] M. J. Bissell,[4] L. M. Coussens,[5] L. R. Lund,[2] Z. Werb[1]; [1]Anatomy, University of California, San Francisco, San Francisco, CA, [2]The Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark, [3]National Institute for Dental and Craniofacial Research, NIH, Bethesda, MD, [4]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, [5]The Cancer Research Institute and Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA

Branching morphogenesis is the process of epithelial ductal invasion and branching into stromal tissue and it requires stromal remodeling, which can be regulated by matrix metalloproteinases (MMPs). We show here that branching morphogenesis of the mouse mammary gland requires MMP-14 (MT1-MMP), its activation of MMP-2 and the cleavage of type I collagen by both enzymes. Mammary glands from mice deficient in MMP-14 had severely reduced epithelial branching, abnormal terminal end bud morphology and extensive collagen deposition. In keeping with MMP-14's ability to activate MMP-2 (gelatinase A) and cleave type I collagen, mammary glands from mice deficient in MMP-2 or with mutated type I collagen genes resistant to collagenolytic MMPs, including MMP-14, also had delayed mammary gland development. However, only composite mutant mammary glands, deficient for MMP-2 and with MMP-14 resistant collagen, had the same pronounced collagen deposition as the MMP-14 deficient mammary glands, suggesting synergy between cleavage of collagen by MMP-14 and MMP-2. Although ductal elongation was diminished in all these cases, proliferation of epithelial cells in the terminal end buds was normal or even increased, suggesting accelerated programmed cell death and lack of generation of a survival signal. Indeed, mice with mutated type I collagen hypersensitive to proteinase degradation also had delayed mammary gland development, suggesting a role for specific type I collagen cleavage fragments in addition to simple removal of collagen. Together, the data suggest that MMP-14 generates specific collagen fragments that mediate survival signals and that the further degradation of collagen by MMP-14-activated MMP-2 is important for path-clearing. We propose that this coordinated degradation of type I collagen by MMPs plays a central role in branching morphogenesis.

1827

**Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) is a Path-Generating Collagenase in Mammary Gland Branching Morphogenesis**

H. Mori, J. Alcaraz, C. M. Nelson, M. J. Bissell; Life Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA

Proper mammary gland morphogenesis and differentiation requires strict temporal and spatial control of cellular function. These processes are coordinated by changes in the structure and composition of the surrounding extracellular matrix (ECM), orchestrated in large part through the action of matrix metalloproteinases (MMPs), which play a key role in degradation and reorganization of ECM as essential components of cell proliferation and migration. MT1-MMP has been shown to play a key molecule in cancer cell invasion and metastasis. During mammary gland development, ductal elongation and branching morphogenesis involve epithelial cell invasion into a collagen- and laminin-rich mammary fad pad, and MT1-MMP is expected to play a role. To dissect the role of MT1-MMP, we used three-dimensional collagen I gels and EpH4 mouse mammary epithelial cells. Whereas stable expression of MT1-MMP enhanced tubular elongation and side branching, its hemopexin domain inhibited

branching presumably by interfering with dimerization of MT1-MMP. Reduction of endogenous MT1-MMP by RNAi expression inhibited both tubular elongation and side branching. Using collagen gels of different stiffness, we show that collagenous activity per se is not essential for branching. In gels with lower stiffness, EpH4 cells branch without MMP activity, but at higher gel stiffness, EpH4 branching requires MMP activity. We thus propose that in vivo, MT1-MMP activity is required for branching mainly to reduce the stiffness of the surrounding matrix to allow the growing end bud to invade the stroma.

1828

**Identification of the Key Control Mechanisms of Mammary Epithelial Branching Morphogenesis**

C. M. Nelson,[1] M. van Duijn,[2] J. L. Inman,[1] D. A. Fletcher,[2] M. J. Bissell[1]; [1]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, [2]Department of Bioengineering, University of California, Berkeley, CA

Branching morphogenesis, the formation of branched epithelial tubes from a preexisting structure, is common throughout development and is responsible for generating the complex architectures of lung airways, kidney collecting ducts, and the salivary and mammary glands. The most fundamental step of branching morphogenesis, branch site initiation, has been found to be regulated by a number of cues, including growth factors, proteases, and morphogens. To determine how these cues are integrated spatially to result in characteristic, organ-specific patterns of branching, we have developed a quantitative three-dimensional micropatterned assay of mammary epithelial branching morphogenesis to define the signals that control the position of branch site initiation. Mouse mammary epithelial cells were engineered to form precisely defined multicellular duct-like structures within gels of collagen type I. Onset of branching morphogenesis was controlled temporally by the exogenous addition of growth factors to stimulate the morphogenic program. We found that these structures branched in characteristic, highly reproducible patterns from specific locations depending on the local microenvironment and the initial geometry of the multicellular structure. We identified the key alterations in signaling and gene expression at sites of branch initiation and found transient upregulation of genes associated with the mesenchymal phenotype. We show that the form of the duct-like structures coordinates signaling from growth factors and morphogens to regulate the position of branch initiation.

1829

**Fibronectin Modulates Mammary Epithelial Cell Morphology during Acinar Differentiation**

C. M. Williams, J. E. Schwarzbauer; Molecular Biology, Princeton University, Princeton, NJ

Mammary epithelial cells (MECs) *in vivo* form branching ducts that end in hollow acini surrounded by a basement membrane. Breast cancer results in derangement of tissue architecture and increased amounts of fibronectin, an extracellular matrix protein normally absent from mammary tissue, is found in the stroma. This correlation between a fibronectin-rich extracellular matrix and aberrant proliferation and morphology may point to a role for fibronectin signaling in early stages of breast oncogenesis. The non-tumorigenic mammary epithelial cell line, MCF-10A, is capable of differentiating into acini *in vitro* when cultured on Matrigel, a reconstituted basement membrane matrix. Unlike MECs *in vivo*, MCF-10A cells express fibronectin and its α5β1 integrin receptor and assemble a fibronectin matrix in monolayer culture. Fibronectin is absent from mammary tissue *in vivo* suggesting that its expression must be down-regulated as acini form. However, fibronectin appears to play a role in the early stages of acini formation since knock-down of endogenous MCF-10A fibronectin using small interfering-RNAs perturbed acinar polarity and lumen formation. Analysis of developing acini by immunofluorescence staining with anti-fibronectin antibodies localized fibronectin to the basal cell surface, on the outside of the acini. Growth on Matrigel, which does not contain fibronectin, resulted in down-regulation of fibronectin production by MCF-10A cells. Within 3 days of plating, little if any secreted fibronectin was detected. To mimic the primary breast tumor microenvironment, fibronectin was added to the Matrigel. In this environment, cells maintained fibronectin expression and formed large, poorly polarized acini that failed to form a hollow lumen. These data show that failure to down-regulate fibronectin expression prevents normal acinar morphogenesis. During cancer progression, increased stromal fibronectin may provide a microenvironment that modulates epithelial cell morphology and disrupts signals required for maintenance of the differentiated state.

1830

**Force-dependent Apoptosis Resistance and Breast Tumorigenesis**

J. L. Leight,[1] N. Zahir,[1] J. N. Lakins,[2] J. Y. Wong,[3] V. M. Weaver[2]; [1]Bioengineering, University of Pennsylvania, Philadelphia, PA, [2]Pathology, University of Pennsylvania, Philadelphia, PA, [3]Biomedical Engineering, Boston University, Boston, MA

Apoptosis resistance plays a key role in malignant transformation and metastasis of the breast. Because cell-extracellular matrix (ECM) interactions regulate mammary epithelial cell (MEC) survival, we have been studying how the ECM could influence malignant transformation and treatment responsiveness of breast tumors. Previously we identified α6β4 integrin dependent activation of NFκB as a key apoptosis resistance mechanism that is up regulated in breast cancers. We showed that the efficacy of NFκB-mediated breast cancer survival is greatly enhanced when MECs are grown within a compliant ECM to form a three-dimensional (3D) tissue-like structure. To test the hypothesis that three-dimensionality and matrix compliance regulate apoptosis resistance and to delineate the underlying mechanisms, we used reconstituted basement membrane cross-linked polyacrylamide gels of defined elastic moduli onto which we cultured MECs as 3D structures. Here we report that MECs form organized acini-like structures on a compliant matrix (140 Pa) and exhibit a markedly enhanced survival phenotype to chemotherapeutic agents and immune receptor activators within 5-6 days. In contrast, on a stiff matrix (60 kPa) MECs fail to undergo morphogenesis and undergo apoptosis readily in response to exogenous death stimuli, even after formation of non-differentiated multi-cellular structures. 3D compliant MEC structures acquire apoptosis resistance coincident with tissue polarity and a blunted ability to stimulate the stress genes AP-1 and SRE. Because we showed that compliance-dependent apoptosis resistance correlates with a failure to induce stress genes in response to apoptotic insults, we conclude that matrix compliance regulates stress-dependent gene expression. Using synthetic 2D and 3D gels of calibrated stiffness we are currently exploring the mechanism whereby matrix compliance and three-dimensionality could regulate stress gene expression to modulate tissue polarity and apoptosis resistance. Supp. DOD W81XWH-05-1-330, HL6438801A1 & CA078731.

1831

**The Role of Rab27b in Breast Cancer Development**

W. Westbroek,[1] O. De Wever,[2] A. Helip Wooley,[1] M. Mareel,[2] M. Huizing,[1] M. Bracke,[2] W. A. Gahl[1]; [1]Medical Genetics Branch, NHGRI, NIH, Bethesda, MD, [2]Laboratory of Experimental Cancerology, Ghent University Hospital, Gent, Belgium

Rabs are ras-like small GTPases that regulate vesicle movement, sorting and secretion. Rab27b is a member of the Rab27 subfamily that shows 72% homology to Rab27a on amino acid level. Rab27b and Rab27a can have the same function in transport and docking of lysosome related organelles in different cell types. Rab27b but not Rab27a has been described as a potential biomarker in breast cancer progression. We prepared stable MCF-7/AZ cells expressing eGFP, eGFP-Rab27a or eGFP-Rab27b and studied the organization of the F-actin cytoskeleton by confocal microscopy and the activation status of Rho GTPases by pull down assay and invasion into Matrigel. We found that cells overexpressing Rab27b, but not Rab27a, show a tremendous change in the organization of the actin cytoskeleton characterized by filopodia-like extensions searching for contact with neighbouring cells. Furthermore, Rab27a and Rab27b localized to vesicular structures; we are now characterizing these vesicles using specific markers. Cells overexpressing Rab27b, but not Rab27a, were invasive into Matrigel. This correlated with a reduction in the activation of the RhoA GTPase, and a stimulation in the activation status of the Ras GTPase as demonstrated with a pull down assay. We did not observe changes in the activation status of the Rac GTPase. Invasion into Matrigel was annihilated by pharmacological inhibitors of PI3K, Akt and MAPK, and by co-transfection of constitutive active mutants of RhoA and dominant negative mutants of Ras and Rac. With these data, we describe for the first time the putative role of a Rab protein (Rab27b) in invasion and actin-reorganization of MCF-7/AZ breast cancer cells. Research on the biological function of up-or down-regulated Rabs in tumor progression could open new perspectives in cancer research.

1832

**Loss of Small Intestinal Villi in Adult Laminin α5 Deficient Mice**

X. Zhen, T. S. Stappenbeck, J. H. Miner; Washington University School of Medicine, St. Louis, MO

The laminin α5 chain is a widely expressed component of both laminin-α5β1γ1 and laminin-α5β2γ1 trimers that are prominent components of basement membranes (BMs) in numerous tissues. *Lama5-/-* mice die perinatally and exhibit multiple developmental defects, such as neural tube closure, digit septation, placentation, lung lobe septation, kidney development, and hair growth. We used a widely expressed laminin α5 transgene (Mr5) to rescue these defects; adult *Lama5-/-*; Mr5 mice appear normal and are viable and fertile. However, Mr5 expression wanes after birth, resulting in greatly reduced levels of laminin α5 in some BMs, including those in the distal small intestine. Normally, the intestinal mucosa that lines the inner surface of the gut tube is organized by repeating units of finger-like villi and adjacent invaginated crypts. In the *Lama5-/-*; Mr5 mice, villi coalesce in the distal small intestine to form cerebroid (like the surface of the brain) or mosaic (individual villi no longer present) patterns. These morphologic patterns strongly resemble villus atrophy observed in human celiac disease and during nematode infection. However, unlike these disease states, there is not inflammatory infiltrate in the small intestines of *Lama5* deficient mice. In the small intestine of wild-type adult mice, epithelial cells proliferate in the base of crypts. Their progeny differentiate into four distinct lineages, three of which, enterocytes, enteroendocrine cells, goblet cells migrate onto and up adjacent villi. The fourth lineage, Paneth cells are retained in the base of the crypt. The epithelium of *Lama5-/-*; Mr5 mice is abnormal and displays alteration in proliferation, Paneth cell differentiation and migration of cells on the villus. Together, these results suggest that laminin α5 and intestinal BMs play crucial roles in organizing crypt-villus architecture, and in regulating the activities of the intestinal epithelial cells.

1833

**Localization of Annexin II and V during Chondrogenesis and Calcification**

T. Minashima,[1] H. Aso,[2] K. Tahara,[3] T. Yamaguchi,[3] S. Takano,[3] S. Ohwada,[1] K. Watanabe,[1] T. Yamaguchi[1]; [1]Cellular Biology Laboratory, Faculty of Agriculture, Tohoku University, Sendai, Japan, [2]Tohoku University, Sendai, Japan, [3]R & D Center, BML Inc., Saitama, Japan

Mesenchymal stem cells have demonstrated the multipotential to differentiate into several cell lineages; osteogenic, chondrogenic or adipogenic lineages. However the exact mechanism of the steps in the pathway leading from an undifferentiated stem cell to a mature chondrocyte are not well understood. A clonal bovine intramuscular preadipocyte (BIP) line has an ability of differentiating to the chondrogenic lineage, because their formed spherical pellets had a chondrogenic morphology and produced typical marker molecules for chondrogenesis, such as cartilage-specific proteoglycans and type II collagen. Under the proteomic approach, one of new spots was identified as annexin II (ANX II) during the chondrogenic differentiation of BIP cells. AXN II as well as AXN V was a marker for terminally differentiated chondrocytes in hyaline cartilage. In the chondrogenic pellet of BIP cells, AXN II increased until 7 days, and disappeared at 28 days, however, AXN V was stained at the periphery until 14 days, and strongly detected also in the center at 28 days. We also investigated expression of AXN II and V in cartilages. In cartilage without calcification, AXN II was detected in chondrocytes and AXN V was detected in chondrocytes or the extracellular matrix surrounding chondrocytes. On the other hand, AXN II and V in cartilage with calcification were strongly stained in the extracellular matrix. ANX II. These results suggest that there is a quite difference in co-expression mechanism of AXN II and V between hyaline cartilage with calcification and the other cartilages, and that the calcification may need the release of AXN II as same as AXN V from cytosole to the extracellular matrix.

1834

**The Self-assembled Network of Type 1 Collagen Revealed by Fibrillogenesis and Topological Studies**

S. Han, W. Losert; Physics, University of Maryland, College Park, MD

Type I collagen is the most abundant protein of connective tissues including tendon, skin and bone .Collagen molecules exhibit the ability to polymerize into fibers (fibrillogenesis), forming the extra cellular matrix. Recent studies of fibrillogenesis of type I normal and mutant collagen (hetero- and homotrimers) revealed a substantial difference in the kinetics of self-assembly, in particular, the absence of lag time in homotrimers. In heterotrimers, a normal S-shaped curve with a typical lag time for nucleating was monitored by turbidity measurements. The origin of this difference and the underlying mechanism of self-assembly are still unknown. We developed a technique for differential fluorescent labeling of collagen which allows us to study self-assembly of collagen and mixture of collagens (e.g. hetero- and homotrimers) using confocal microscopy to visualize collagen fiber formation in 4D(3space,time) while the network self-assembles. We observed that hetero- and homotrimers were co-localized during fibrillogenesis, despite their very different fibrillogenesis kinetics. Turbidity measurements of fibrillogenesis of the mixture of

hetero- and homotrimers showed the S-shaped curve with lag time typical for heterotrimers. The finer and straighter network structure of homotrimers than normal heterotrimers in images of confocal microscopy was observed. We characterized the structural homology of the 3D network through Betti numbers that are robust to variations in scale, primarily describing the connections between different components comprising the geometric structure. The number of Betti numbers equals the dimension of the structure being analyzed.

1835

**Conditional Deletion of Focal Adhesion Kinase (FAK) Perturbs Matrix Organization in the Developing Mouse Eye**

H. Beggs; Ophthalmology and Physiology, University of California, San Francisco (UCSF), San Francisco, CA

Focal adhesion kinase (FAK) is a key mediator of integrin signaling and regulates cell spreading, migration and organization of the extracellular matrix (ECM). However, early lethality of the complete FAK knockout has precluded direct examination of the physiological relevance of FAK signaling in developing systems. Here, FAK has been conditionally deleted from the developing mouse eye using a Nestin-Cre and Six3-Cre mouse line. The Nestin-Cre line results in deletion of FAK protein from the lens and retina, whereas the Six3-Cre line results in deletion of FAK protein from the retina only. Deletion of FAK from the lens and retina results in significant eye abnormalities, including retinal lamination defects, microphthalmia (small eyes), and complete lens degradation. Interestingly, deletion of FAK from the retina alone resulted in similar defects in retinal layering, yet the presence of a normal lens completely rescued defects in eye size. This suggests that faulty lens development is responsible for stunted eye growth in these animals. In order to determine if matrix organization defects were responsible for the phenotype, two prominent BMs were examined: the lens capsule BM organized by lens fiber and epithelial cells and the retinal inner limiting membrane organized on the endfeet of Muller glial cells. Both were strikingly perturbed at developmental stages immediately preceding gross defects. These results indicate that activation of FAK signaling is an important intracellular step in mediating extracellular organization of the ECM into biologically functional BMs. This is a vital role for FAK signaling that is frequently overlooked in cell culture studies. Interestingly, these eye-related abnormalities are often seen in children with congenital muscular dystrophy (also known as muscle-eye-brain disorders), and are thought to result from defects in cell-matrix binding.

1836

**Slit and Robos Coordinate Cardiac Cell Adhesion and Positioning During *Drosophila* Heart Tube Assembly**

S. G. Kramer, E. Santiago-Martinez, N. H. Soplop; Pathology and Laboratory Medicine, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ

Heart tube assembly requires the precise arrangement of precursor cells on either side of the embryo that coordinately migrate to meet their counterparts at the midline. Little is known about the mechanisms that organize cardiac precursors, once specified, into this complex structure. We analyzed the role of Slit, a secreted extracellular matrix protein and its transmembrane receptors Roundabout (Robo) and Roundabout2 (Robo2) during morphogenesis of the *Drosophila* heart tube, a process analogous to early heart formation in vertebrates. During heart tube assembly, two types of progenitor cells align into rows and coordinately migrate to the midline of the embryo where they merge to assemble a linear heart tube. Here we present that cardiac-specific expression of Slit is required to maintain adhesion between cells within each row during dorsal migration. Moreover, differential Robo expression determines the relative distance each row is positioned from the dorsal midline. The innermost cardioblasts express only Robo, whereas the flanking pericardial cells express both receptors. Removal of *robo2* causes pericardial cells to shift towards the midline, whereas ectopic *robo2* in cardioblasts drives them laterally resulting in an unfused heart tube. We propose a model in which migrating cardiac progenitors intrinsically organize their own assembly into a linear heart tube through secretion of Slit and differential expression of Robo receptors. These findings establish Slit and Robo receptors as critical molecules required for the assembly of vascular tissues.

1837

**Modulation of Renal Proximal Tubulogenesis by the Mammalian Target of Rapamycin**

M. L. Taub; Biochemistry Dept, State University of New York at Buffalo, Buffalo, NY

Tubules may arise during branching morphogenesis through several mechanisms including wrapping, budding, cavitation and cord hollowing. In this report we present evidence that is consistent with renal proximal tubule formation through cord hollowing. Primary rabbit renal proximal tubule cell cultures were initiated in matrigel. Cultures containing developing nephrons were incubated in medium containing Lucifer Yellow, a substrate of the p-Aminohippurate transport system (in the renal proximal tubule, Lucifer Yellow is transported intracellularly by a basolateral p-aminohippurate (pAH) transport system, and is subsequently secreted into the lumen of the nephron). Pockets of lumens filled with Lucifer Yellow were observed within developing cords of the rabbit renal proximal tubule cells in matrigel. The observation of Lucifer Yellow accumulation suggests that the developing nephrons were functionally polarized. Consistent with such polarization, Triticum vulgare (a lectin which binds apically in the rabbit renal proximal tubule) was observed to bind to the luminal membranes within pockets of Lucifer yellow filled lumens. Both EGF and HGF caused an increase in the frequency of tubule formation. PD98059 (an inhibitor of MAPK) inhibited the frequency of tubule formation, unlike Wortmannin (an inhibitor of PI3K). Although the frequency of tubule formation was inhibited by PD98059, this drug had no effect on the process of cavitation. In contrast, rapamycin, an inhibitor of mTOR, prevented cavitation, without affecting the frequency of tubule formation. These results suggest the involvement of MAP kinase in the initiation of the process of tubule formation, while mTOR is involved in the formation of functional renal proximal tubules.

1838

**Elastic System Fibers of Rat Molar Periodontal Ligament: Immunohistochemical Study**

T. Sawada,[1] S. Inoue[2]; [1]Ultrastructural Science, Tokyo Dental College, Chiba, Japan, [2]Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada

Among elastic system fibers of the periodontal ligament of the tooth, oxytalan fibers have been well characterized. However, the localization and nature of elastin-containing fibers, that is, elaunin and elastic fibers, remain to be clarified. In this study, the localization of elastin and fibrillin, major proteins of elastin-containing fibers, were immunohistochemically examined in the periodontal ligament of rat molars. With immunoperoxidase staining at the light microscope level, the distribution of elastin was not uniform throughout the ligament. Elastin-positive

structures were often concentrated in close vicinity of blood vessels in the ligament in areas such as, for example, the middle to apical region of the distal half of the distal root. In contrast, fibrillin was more widely distributed throughout the ligament. The pattern of its distribution was comparable to that of the reported distribution of oxytalan fibers. Fibrillin was also seen concentrated in areas of accumulation of small blood vessels. Immunoelectron microscopy showed that approximately 1 µm wide bundles, often localized close to blood vessels, of fibrillin-positive microfibrils contained small amounts of elastin. It clearly indicated that these entities were elaunin fibers. Mature elastic fibers were not found throughout the ligament. Thus, the elastic system fibers of the rat molar periodontal ligament are composed of only oxytalan and elaunin fibers. In addition to the well known role of the elastic system fibers of supporting the tooth, these fibers are also likely to have the role of protecting the vascular system of the ligament from the force of stress/strain caused by mastication and maintaining its integrity.

1839

**Immobilization of Exogenous Ligands in 3D Matrices**

J. A. Hubbell; Integrative Biosciences Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

Our objective was to develop extracellular matrix analogs that could be engineered to quantitatively incorporate adhesion ligands and growth factors in 3D. We have developed two such model systems, which will be presented. First, we have developed a scheme by which to incorporate exogenous ligands in fibrin matrices during fibrin coagulation. The ligands are either synthesized or expressed as fusions with the sequence NQEQVSP, which incorporates a substrate for the coagulation transglutaminase factor XIIIa. During coagulation, this linker is then covalently grafted within the fibrin matrix. When the 6th Ig-like domain of L1 was thus grafted within fibrin, as a ligand for integrin alpha v beta 3, the angiogenic potential of the fibrin matrix was dramatically increased. When vascular endothelial growth factor (VEGF) was thus grafted within fibrin, it induced potent angiogenesis, however also a more mature angiogenesis than was observed with free VEGF. As a second approach, synthetic analogs of fibrin and collagen were developed, namely gels formed by crosslinking vinyl sulfone-functionalized polyethylene glycol and cysteine-terminated peptides. The peptides were designed as substrates for matrix metalloproteinases (MMPs), permitting degradation of the gel structure under the proteolytic activity of migrating cells. Dermal fibroblasts within such matrices were able to spread in 3D and migrate, and their migration could be enhanced by up-regulators of MMP expression (such as TNF alpha) and inhibited by protease inhibitors. Such synthetic extracellular matrix analogs may thus prove useful for studies of cell migration and invasion.

## Cadherins (1840-1860)

1840

**Differential Expression of *Cdh23* Isoforms in the Cochlea and Retina of Wild Type and *Waltzer* Mice**

A. Lagziel, Z. M. Ahmed, L. M. Peters, T. B. Friedman, R. J. Morell; Laboratory of Molecular Genetics, National Institute on Deafness and Other Communication Disorders National Institutes of Health, Rockville, MD

Mutant alleles of *CDH23* are associated either with deaf-blindness with vestibular dysfunction (Usher syndrome type 1, USH1D), or isolated deafness (DFNB12; Bork et al., 2001). In *waltzer (v)* mice, mutant alleles of *Cdh23*(predicted null alleles and therefore thought to model USH1D) cause deafness and vestibular dysfunction but not blindness. We recently reported the spatiotemporal expression pattern of cadherin 23 during development of the inner ear in wild type and in $v^{6J}$ *waltzer* mice (Lagziel et al., 2005). In addition we reported several short isoforms of *CDH23*/*Cdh23* in the wild type, which are also present in the homozygous $v^{6J}$ mouse mutants and are unaffected by this mutant allele. Here we report an analysis of the relative expression levels of alternative transcripts of *Cdh23* in wild type and $v^{6J}$ mice using semi-quantitative real time PCR assays and RNA from cochleae and retinae from different developmental time points. We found that the *Cdh23* isoforms differ in their temporal and spatial patterns of expression. We also found that certain isoforms were down-regulated in the presence of the $v^{6J}$ allele, while other isoforms were up-regulated. The pattern of up or down-regulation was dependant on the tissue examined (retina or cochlea), and on the developmental stage. The results of this study suggest that a correlation of mutant genotype to phenotype in mice and humans will not depend on a model of a single cadherin 23 isoform, but on the resolution of function for each of the *Cdh23* isoforms.

1841

**Cadherin-11 is Required for Otolith Growth in Zebrafish**

S. G. Babb, J. A. Marrs; Nephrology, Indiana University Medical Center, Indianapolis, IN

Fish otoliths are crystals formed of calcium carbonate and organic matrix and are involved in balance and hearing. Cadherins are transmembrane cell-cell adhesion molecules; In zebrafish, we have found that cadherin-11 (Cdh11, OB-cadherin, VN-cadherin) is localized to otoliths, to puncta within the otic lumen (presumably vesicles), and to the underlying neuroepithelium, suggesting a role for cadherin-11 in otolith growth and biomineralization. To investigate the role of cadherin-11 in otolith formation, we generated cadherin-11 knockdowns using morpholino antisense oligonucleotides. Three independent morpholinos made against cadherin-11 produced the same phenotype. In cadherin-11 knockdowns, little or no Cdh11 protein expression was detected, and significantly, otoliths were small or absent. When present, the correct number of otoliths was found in knockdowns, but their shape was more spherical, rather than the flattened shape of otoliths found in control embryos. Also, otoliths were not tightly tethered to the sensory epithelium. Starmaker, a protein that controls crystal size and lattice formation, was absent in cadherin-11 knockdowns examined. Together, these data show that cadherin-11 is necessary for normal otolith growth in zebrafish. These effects suggest that cadherin-11 may be required to create the proper microenvironment within the otic vesicle for otolith seeding and aggregation. Additionally, cadherin-11 homotypic binding may function in vesicle aggregation during otolith formation and in tethering the otolith to the underlying sensory epithelium.

1842

**A Novel Mechanism for Invasion and Intracerebral Migration of Brain Tumours**

D. Maret, D. R. Colman, R. F. Del Maestro; Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, PQ, Canada

Classic cadherins mediate homophilic, calcium-dependent intercellular adhesion. The prodomain of N-cadherin is normally cleaved intracellularly rendering the mature protein competent to mediate adhesion when it is brought to the cell surface. N-cadherin has been shown to promote cell

motility in cancer cells. Our results indicate that uncleaved pro-N-cadherin accumulates at the cell surface in more aggressive glioma and medulloblastoma cells lines compared to less aggressive cell lines, as determined by measuring the surface ratio of pro-N-cadherin to mature N-cadherin. Since total N-cadherin expression has been shown to correlate with increased motility and pro-N-cadherin lacks adhesive function, we hypothesized that loss of adhesion due to aberrant surface expression of the pro-protein may serve as a molecular mechanism for enhanced motility of brain tumour cells. An N-cadherin mutant was engineered where the prodomain could not be cleaved by endogenous proteases but would be cleaved by serum coagulation factor Xa at the cell surface. To test the effect of expression of N-cadherin precursor protein on cellular behaviours, such as migration and invasion, this construct was transfected into the least aggressive glioma and medulloblastoma cell lines. Our results show that pro-N-cadherin surface expression promotes cell migration in wound-healing assays, and increases invasion into a type I collagen matrix, compared to wild-type cells or cells transfected with mock vector. These effects on cell motility can be abrogated upon treatment with factor Xa, presumably via cleavage of the prodomain and activation of the adhesive function of mature N-cadherin. Our work strongly suggests that the abnormal expression of pro-N-cadherin on the surface of certain brain tumour cells plays a pivotal role in augmenting cell migration by compromising adhesive cadherin function of mature N-cadherin.

1843

**Regulation of Neural Crest Migration by Protocadherin-mediated Cell Adhesion**

J. Bononi, R. Bradley; Cell Biology and Neuroscience, Montana State University, Bozeman, MT

Neural crest cells originate along the dorsal neural tube in vertebrate embryos, then delaminate and migrate away from the neural tube to form the majority of the peripheral nervous system (PNS). Cell adhesion molecules are thought to participate in neural crest migration, contributing to both the delamination and subsequent re-aggregation of the neural crest cells to form the various neural crest derivatives. To identify cell adhesion molecules involved in the formation of the PNS we screened an embryonic chicken dorsal root ganglia (DRG) library for members of the protocadherin family, resulting in the isolation of chicken protocadherin-1 (cPcdh1). In the trunk, cPcdh1 is expressed in migrating neural crest cells and in the DRG, sympathetic ganglia (SG) and Schwann cells along the ventral root (VR). Within the DRG, cPcdh1 is first detected as neural crest cells begin to coalesce and is initially restricted to the perimeter, where it colocalizes with undifferentiated and mitotically active cells. This suggests that cPcdh1 may function as a migration arrest signal to localize nascent neural crest cells to the margins of the DRG. To assess the function of cPcdh1, full-length and dominant-negative cPcdh1 constructs were ectopically expressed in neural crest cells by in ovo electroporation. Results indicate that over-expression of cPchd1 alters the distribution of neural crest cells in the PNS, with an increase in the percentage of neural crest cells that migrate to the DRG at the expense of cells that migrate to the SG or VR. Conversely, when cPcdh1 function is inhibited, by ectopic expression of a dominant-negative construct, a greater percentage of neural crest cells are found in the SG and VR. These results suggest that cPcdh1-mediated cell adhesion plays an important role in neural crest cell migration arrest during vertebrate PNS formation.

1844

**Nuclear Localization Signals (NLSs) of E-cadherin Transcriptional Repressor Snail**

H. Ko,[1] K. Kim,[1] H. Kim,[2] X. Li,[3] S. Weiss,[3] J. Yook[2,3]; [1]Department of Biochemistry and Molecular Biology, BK21 Project for Medical Science, College of Medicine Yonsei University, Seoul, Republic of Korea, [2]Department of Oral Pathology, BK21 Projects for Medical Science, College of Dentistry Yonsei University, Seoul, Republic of Korea, [3]Division of Molecular Medicine and Genetics, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, University of Michigan, Michigan, MI

The E-cadherin transcriptional repressor, Snail, plays a critical role in driving the epithelial-mesenchymal transition programs that mark gastrulation as well as invasion of cancer cells. Recent data suggested that Snail is phosphorylated by glycogen synthase kinase-3β (GSK3-β), resulting in β-Trcp mediated ubiquitination and proteasomal degradation. Accordingly, Wnt signaling inhibits Snail phosphorylation and consequently increases Snail protein levels. In the present study, we have examined the roles of a Snail nuclear localization motif. Typical NLS sequences is located at the N-terminal of Snail overlapping with SNAG domain (residue 8-16) and in front of zinc-fingers (residues 151-152) and are a bipartite NLS motif and a basic cluster NLS motif, respectively. These sequences are highly conserved from xenopus to human. Mutational inactivation of these NLS signals resulted in decreased expression level of nuclear and total Snail protein suggesting NLSs are essential for proper function. NLS mutations resulted in decreased expression of nuclear and cytoplasmic Snail, with corresponding attenuation of Snail repressor activity using a reporter gene assay with an E-cadherin proximal promoter. LiCl treatment increased the expression level of the cytoplasmic fraction of Snail suggesting that cytoplasmic fraction of Snail is rapidly phosphorylated and degraded by GSK3-β. Confocal microscopic examination also showed increased cytoplasmic localization of NLS mutants. These results indicate that the highly conserved N-terminal SNAG domain and the zinc finger domain of Snail function as nuclear localization signals and that Snail expression can be regulated by subcellular localization and phosphrylation by GSK3-β.

1845

**Developing Nanostructured Surfaces to Investigate the Role of Cadherins in Cell Adhesion and Cell Motility**

T. Wolfram,[1,2,3] T. Schoen,[1,3] E. Bock,[2] M. Arnold,[3] T. Lohmueller,[2] P. Girard,[3] J. P. Spatz[1,2,3]; [1]Institute for Molecular Biophysics, The Jackson Laboratory, Bar Harbor, ME, [2]Dept. New Materials and Biosystems, Max-Planck-Institute for Metals Research, Stuttgart, Germany, [3]Dept. Biophysical Chemistry, University of Heidelberg, Heidelberg, Germany

$Ca^{2+}$-dependent cell-cell contacts mediated via homophilic interactions between members of the Cadherin-family are key events in regulating structural integrity of cells and tissue. It becomes more and more obvious that cadherins facilitate not only those specific events, but also control a wide variety of cellular behaviours. They are playing instructive rolls for cell and tissue polarization. They regulate different types of cell movement like cell sorting, cell migration and cell rearrangements. In order to investigate and to mimic positioining of single molecules of N-Cadherin and Cadherin-8 on the cell membrane, we designed a hexagonally close-packed rigid template of gold nano-dots based on Micellar Nanolithography. These gold dots are structured by self-assembly of diblock co-polymers and positioned uniformly or as a gradient over different substrate spacings. The gold dots are biofunctionalised via a Mono-Thiol-NTA group with His-tagged N-Cadherin (Cadherin-2) and Cadherin-8. The glass area between biofunctionalised gold dots is covered with Polyehtyleneglycol (PEG) to circumvent unspecific binding of the cells to the glass surface, giving us the opportunity to exactly control the chemical environment on the cover slips. In the present study, we applied a variety of

different proneural cell lines (B35, PC12, SHSY-5Y, N2a) and primary neuronal cells on these surfaces. Most of the cell lines bind to those substrates and are able to build cellular protrusions. Distances higher than 58nm resulting in less cell adhesion and leading to a higher grade of cell motility. Furthermore we would like to address the questions how the molecular composition of cytoskeleton components like actin, are influenced by different nanometer-patterend surfaces. In general our unique technique enables us to probe proteins involved in cell adhesion, cell signaling, cell survival and cell morphology in a structurally controlled chemical environment.

1846

**Partial Cadherin Unfolding Is a Prerequisite for Adhesive Dimerization**

R. B. Troyanovsky, O. Y. Laur, S. M. Troyanovsky; Dermatology, Washington University Medical School, St. Louis, MO

In this study we compared E-cadherin dimerization on the surface of living cells with how the same process unfolds on agarose beads. In both cases, dimerization was monitored by the same site-specific cross-linking assay. Our work showed that on the bead surface, under native conditions, E-cadherin produced predominantly low affinity (weak) dimers that immediately dissociated after the depletion of calcium ions. However, in either a slightly acidic buffer (pH 5) or in the presence of cadmium ions, E-cadherin produced high affinity (strong) dimers. These dimers were unable to dissociate upon calcium depletion. Both types of dimers apparently had similar structures, and their formations were abolished by the same point mutations (W156A or mutations of the EC1/EC2 calcium-binding sites). Remarkably, only strong dimers were revealed on the cell surface under normal culture conditions. These dimers correspond to the adhesive dimers previously identified in coimmunoprecipitation experiments. The formation of weak dimers was detected on the plasma membrane only when cells had been fragmented by homogenization and E-cadherin was immunoclustered. Taken together, our study uncovers a previously unknown cellular process involving EC1 domain unfolding and leading to the formation of strong cadherin adhesive dimers.

1847

**Regulation of N-Cadherin Adhesion Strength by PIP2 Generated by PIP5KIγ**

T. Y. El Sayegh,[1] P. Arora,[1] C. Laschinger,[1] R. Anderson,[2] P. Janmey,[3] C. McCulloch[1]; [1]CIHR Group in Matrix Dynamics, University of Toronto, Toronto, ON, Canada, [2]Pharmacology, University of Wisconsin, Madison, WI, [3]Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA

Intercellular adhesion is critical for tissue homeostasis but the role of phosphoinositides in regulating the localization and function of actin binding proteins involved in mediating cadherin function has not been examined. We used the donor-acceptor model and a recombinant protein consisting of the extracellular domain of N-cadherin-mouse IgG Fc region, to examine the regulation of N-cadherin junctions by phosphatidylinositol 4,5-biphosphate ($PIP_2$) and gelsolin. $PIP_2$ was generated locally at sites of N-cadherin-mediated intercellular adhesion and was an important regulator of adhesion strength. $PIP_2$-specific antibodies and a GFP-tagged construct of the pleckstrin homology domain of phospholipase Cγ, showed $PIP_2$ enrichment at sites of early N-cadherin ligation. Immunostaining for type I phosphatidylinositol 4-phosphate 5 kinases (PIP5KI) showed that the PIP5KIγ isoform spatially associated with N-cadherin coated beads. Dominant negative PIP5KIγ blocked $PIP_2$ enrichment at sites of N-cadherin ligation and also reduced adhesion strength compared to wt-PIP5KIγ and untransfected controls as determined by shear wash-off assay. Cells incubated with a cell-permeant peptide that competes with the $PIP_2$ binding region of gelsolin showed significantly reduced adhesion strength compared with an irrelevant control peptide ($p<0.05$). This finding underlined the importance of $PIP_2$-induced uncapping of actin filament barbed ends for adhesion. Gelsolin-null fibroblasts transfected with full length gelsolin actin severing mutants (characterized by 80% reduction in severing efficiency but retaining PIP2 binding and barbed end interaction) showed similar adhesion strength as wild-type gelsolin-transfected controls. Collectively these results indicate that transient, localized generation of $PIP_2$ by PIP5KIγ may regulate intercellular adhesion strength in fibroblasts, in part by a mechanism utilizing gelsolin uncapping of actin filaments.

1848

**The Effect of Neurocan on N-cadherin Function is Mediated by Fyn tyrosine kinase**

J. Jiang, J. Lilien, J. Balsamo; Biological Sciences, The University of Iowa, Iowa City, IA

Neurocan is a chondroitin sulfate proteoglycan in the aggrecan, versecan, brevican family, that is highly enriched in the nervous system and thought to act as an axonal guidance cue. Binding of neurocan, or a recombinant N-terminal fusion protein, to its cell surface receptor initiates a signal cascade that results in disruption of the complex of proteins associated with the cytoplasmic domain of N-cadherin, tyrosine phosphorylation of beta-catenin, and consequent loss of N-cadherin function. We now present evidence that the effect of neurocan on N-cadherin function depends on the integrity of lipid rafts and that Fyn, a member of the Src family of tyrosine kinases, plays a major role in neurocan initiated signaling. Disruption of lipid rafts by cholesterol depletion results in loss of response to neurocan. Additionally, inhibition of Fyn catalytic activity by the tyrosine kinase inhibitor PP1 eliminates the effect of neurocan. When the association of Fyn with lipid rafts is prevented by the palmitolation inhibitor 2-bromopalmitate, the amount of Fyn associated with N-cadherin increases, with a concomitant decrease in beta-catenin association with cadherin and loss of N-cadherin function. Loss of beta-catenin from the N-cadherin complex as a result of 2-bromopalmitate can be prevented by inhibiting Fyn activity with PP1, suggesting that the association of Fyn with lipid rafts modulates the ability of Fyn to phosphorylate beta-catenin. Indeed, Fyn specifically phosphorylates beta-catenin on tyrosine residue 654. Phosphorylation of this residue is known to result in a dramatic reduction in the affinity of beta-catenin for cadherin. Consistent with this, introduction of the beta-catenin mutant Y654F into neural retina cells effectively blocks the cells response to neurocan.

1849

**Rhoa Gtpase Regulates M-cadherin Activity and Myoblast Fusion**

S. Charrasse,[1] F. Comunale,[1] Y. Grumbach,[1] A. Blangy,[1] F. Poulat,[2] C. Gauthier-Rouviere[1]; [1]CRBM-CNRS, Montpellier, France, [2]IGH-CNRS, Montpellier, France

The Rho family of GTP-binding proteins plays critical roles during myogenesis induction. To elucidate their role later during myogenesis, we have analyzed RhoA function during myoblast fusion into myotubes. We find that RhoA activity is rapidly and transiently increased when cells are shifted into differentiation medium and then is decreased until myoblast fusion. RhoA activity must be down-regulated to allow fusion, as

expression of a constitutively active form of RhoA (RhoAV14) inhibits this process. RhoAV14 perturbs the expression and localization of M-cadherin, a member of the $Ca^{2+}$-dependent cell-cell adhesion molecule family that has an essential role in skeletal muscle cell differentiation. This mutant does not affect N-cadherin and proteins involved in myoblast fusion, β1-integrin and ADAM12. Active RhoA induces the entry of M-cadherin into a degradative pathway and thus decreases its stability in correlation with the monoubiquitination of M-cadherin. Moreover, p120 catenin association with M-cadherin is decreased in RhoAV14-expressing cells, which is partially reverted by the inhibition of the RhoA effector Rho-associated kinase ROCK. ROCK inhibition also restores M-cadherin accumulation at the cell-cell contact sites. We propose that the sustained activation of the RhoA pathway inhibits myoblast fusion through the regulation of p120 activity, which controls cadherin internalization and degradation.

1850

**a-Actinin Links LPP, but not Zyxin, to Assembly of Cadherin-Based Junctions**

M. D. H. Hansen, M. C. Beckerle; Huntsman Cancer Institute, Salt Lake City, UT

Physiologically relevant cell-cell adhesion requires tethering of cadherin adhesion receptors to actin networks. Cadherin engagement results in dramatic changes in actin dynamics and organization. The cytoplasmic domain of cadherin binds a series of proteins that facilitate actin binding, including α-actinin. Interestingly, the related proteins zyxin and LPP contain functional α-actinin binding sites and bind members of the VASP family of actin regulatory proteins. It has been proposed that zyxin serves as an adaptor to recruit VASP into cadherin complexes in order to drive actin rearrangements. In support of this hypothesis, zyxin colocalizes with VASP at cell-cell junctions. VASP is required for normal cell-cell junction formation, as are the VASP binding motifs of zyxin and LPP. Here we examine whether α-actinin docks zyxin and/or LPP to cadherin complexes. We find that LPP, but not zyxin, requires an α-actinin binding site to drive cell-cell adhesion. Further, dominant negative LPP, but not zyxin, mutants prevent the incorporation of α-actinin into cadherin-actin networks. Thus, α-actinin, through LPP, likely docks VASP family members to cadherin complexes. These results provide a mechanism for linking VASP-mediated regulation of actin dynamics to cell-cell adhesion.

1851

**Zyxin and LPP-VASP Complexes Drive Cell-Cell Adhesion in Response to Rac1**

M. D. H. Hansen, M. C. Beckerle; Huntsman Cancer Institute, Salt Lake City, UT

Cadherins mediate cell-cell adhesion by linking cell junctions to actin networks. Mechanisms of cadherin-actin network assembly remain poorly understood. We examine how zyxin and the related protein LPP contribute to cadherin-actin network formation. Using a quantitative assay for cell-cell adhesion, we demonstrate that zyxin and LPP function to increase the rate of early cell-cell junction assembly. This function is inhibited by the LIM domains and requires intact ActA repeats, which bind VASP family members. Constitutively active zyxin or LPP mutants that lack LIM domains drive increased VASP activity at cell-cell junctions, as measured by increased incorporation of VASP into the actin cytoskeleton and by displacement of capping protein. Recruitment of zyxin/LPP to cell-cell junctions is stimulated by Rac1, suggesting a mechanism by which signaling pathways might control localization and activity of zyxin/LPP-VASP complexes. We propose a model in which zyxin/LPP LIM domains serve as a regulatory switch that exerts local control over VASP activity and thus influences strengthening of cell junctions.

1852

**Immediate-Early Dynamics at E-cadherin Contacts**

T. D. Perez,[1] M. Tamada,[2] S. Yamada,[1] M. P. Sheetz,[2] W. J. Nelson[1]; [1]Molecular and Cellular Physiology, Stanford University, Stanford, CA, [2]Biological Sciences, Columbia University, New York, NY

The Ca++-dependent cell-cell adhesion protein E-cadherin is an early regulator of cell-cell contacts necessary for establishing contacts and allowing epithelial cells to polarize. Though many regulators of E-cadherin adhesion have been found, the mechanisms and dynamics of E-cadherin adhesion are still not well understood during early cell-cell contact formation. To better study the dynamics of proteins at E-cadherin contacts, we utilized small, polystyrene beads coated with the extracellular domain of E-cadherin to initiate cadherin-mediated contacts. Coated beads were placed on cells using a laser trap, and the dynamics of E-cadherin recruitment were determined with respect to Rac, Akt-PH domain, actin, and Arp3 localization at the beads. We find that maximal E-cadherin localization occurs in one of two modes. A fast mode of cadherin accumulation completes within 1 minute and is concurrent with Rac, actin, and PH-domain accumulation. A slow mode of cadherin accumulation occurs over 3 minutes and precedes the initiation of Rac, actin, and PH-domain accumulation by about 2 minutes. E-cadherin fluorescence remained stable after maximal accumulation, but Rac and PH-domain were rapidly lost at contact sites. Additionally, we find no dependence of early cadherin or Rac accumulation on PI3K activity. The results show that early E-cadherin accumulation can be independent of Rac1 and PI3K signaling and allow us to propose new models of how E-cadherin may function as a cell-cell contact receptor.

1853

**E-cadherin Knockdown Disrupts Establishment but not Maintenance of Cell Polarity in MDCK Cells**

C. T. Capaldo,[1] I. G. Macara[2]; [1]Pharmacology, University of Virginia, Charlottesville, VA, [2]University of Virginia, Charlottesville, VA

E-cadherin is a single pass transmembrane glycoprotein that forms calcium dependent, homophilic intercellular adhesions in epithelial cells. These contacts are key regulators of cell adhesion and differentiation, including the assembly of intercellular Tight Junctions (TJs). TJs act as a permeability barrier between cells and maintain cell polarity by preventing diffusion between apical and basal-lateral membrane compartments. TJ formation is stimulated by cell-cell contact, but the mechanisms involved are poorly understood. In the following study, Madin-Darby canine kidney (MDCK) cells were selectively depleted of E-cadherin by RNAi. Surprisingly, this had little effect on the protein levels or localization of several markers of AJs (Adherens Junctions) and TJs. Also, immunofluorescence staining with the apical marker gp135 and the basal-lateral marker Na+ K+ ATPase showed no loss of apical-basal polarity. Fluorescence Recovery After Photobleaching (FRAP) analysis in MDCK cells stably expressing YFP-ZO-1 indicates that E-cadherin knockdown does not alter the apparent stability of tight junction complexes. However, E-cadherin knockdown cells failed to properly establish cell polarity. In response to calcium switch, knockdown cells exhibited severely suppressed trans-epithelial resistance and delayed localization of TJ and AJ markers. These studies indicate that E-cadherin functions in the establishment of cell polarity, but that wild-type levels of E-cadherin are not required for the maintenance of Tight Junctions and cell polarity.

1854

**N-cadherin Adhesion and Cell Signaling Regulates Polarity of Migrating Vascular Smooth Muscle Cells**

P. J. B. Sabatini,[1] M. Zhang,[2] M. P. Bendeck,[1] B. L. Langille[1,2]; [1]Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada, [2]University Health Network, Toronto, ON, Canada

Signaling associated with cellular adhesion is a key process for cell migration that promotes cytoskeletal reorganization and couples anterior protrusion and attachment with posterior retraction. Accordingly, anterior activation of integrin-mediated cell-substrate adhesion activates Cdc42 to reorient the microtubule-organizing center (MTOC) towards the direction of migration. N-cadherin polarizes to the posterior-lateral margins of many wound-edge cells and can regulate GTPases; therefore, we hypothesized that N-cadherin also regulates MTOC reorientation in a scrape-wound assay. Treatment with an N-cadherin blocking antibody, or a small peptide that interferes with N-cadherin-mediated cell adhesion, disrupted polarization of the MTOC to the anterior side of the nucleus. Cdc42 was activated by wounding and transfection of a GFP-tagged wild-type Cdc42 plasmid confirmed anterior localization of wound edge cells; however, blocking N-cadherin with antibody had no effect on Cdc42 activity and anterior localization after wounding, thus N-cadherin may exert effects that are independent of Cdc42. The N-cadherin antibody disrupted actin reorganization in wound-edge cells and tracking of microtubules to the anterior of the cell along microfilament bundles was also disrupted. Wounding also rapidly suppressed RhoA activity, a target of N-cadherin signaling, and current experiments are exploring the influences of N-cadherin on RhoA and cytoskeletal reorganization in these cells.

1855

**A Role for N-cadherin in Endothelial Cell Migration via PDGF-R Signaling**

C. Theisen, K. R. Johnson, M. J. Wheelock; Oral Biology, University of Nebraska Medical Center, Omaha, NE

Tumor cells recruit endothelial cells from existing capillaries in order to establish their own vascular system via angiogenesis. Endothelial cells express two classical cadherins, VE-cadherin and N-cadherin. VE-cadherin is localized to the cell-cell junctions in endothelial cells while N-cadherin is not a component of the junctional complex. Using a stable siRNA system we were able to knock-down N-cadherin expression in the immortalized human microvascular endothelial cell line HMEC-1. N-cadherin knock-down cells have impaired ability to form tubes on matrigel in an *in vitro* angiogenesis assay and decreased motility in a transwell motility assay. Using phage display we also discovered a novel binding partner for β-catenin known as NHERF-2. We have mapped regions required for association of both proteins to PDZ II of NHERF-2 and a PDZ binding motif at the very carboxy-terminus of β-catenin. The PDZ I domain of NHERF-2 is known to bind PDGF-Rβ and this interaction leads to enhanced cell spreading and motility. PDGF signaling is also involved in tube formation of microvascular endothelial cells. We show that β-catenin and N-cadherin are in a complex with NHERF-2 and PDGF-Rβ at membrane ruffles using immunoprecipitation and immunofluorescence studies on HT1080 cells expressing NHERF-2 and PDGF-Rβ. HMEC-1 cells are less motile and have impaired tube formation in the presence of a PDGF-R specific inhibitor. These studies implicate N-cadherin and β-catenin in endothelial cell migration and tube formation via PDGF-R mediated signaling through the scaffolding molecule NHERF-2.

1856

**Expression of Inappropriate Cadherins by Epithelial Tumor Cells Promotes Endocytosis and Degradation of E-cadherin via Competition for P120 Catenin**

S. Mandal, M. Maeda, K. R. Johnson, M. J. Wheelock; Oral Biology, University of Nebraska Medical Center, Omaha, NE

Cadherin cell-cell adhesion proteins play an important role in modulating the behavior of tumor cells. E-cadherin serves as a suppressor of tumor cell invasion, and when tumor cells turn on the expression of a non-epithelial cadherin, they often express less E-cadherin, enhancing the tumorigenic phenotype of the cells. Here we show that when A431 cells are forced to express R-cadherin they dramatically down regulate expression of endogenous E-cadherin and P-cadherin. In addition, we show that this down regulation is due to increased turnover of the endogenous cadherins via clathrin dependent endocytosis. p120 catenin binds to the juxtamembrane domain of classical cadherins and has been proposed to regulate cadherin adhesive activity. One way p120 catenin may accomplish this is to serve as a rheostat to regulate the levels of cadherin. Here we show that the degradation of E-cadherin in response to expression of R-cadherin is due to competition for p120 catenin.

1857

**Desmosome Disassembly and Desmoglein Endocytosis are Coordinated Responses to Pemphigus Autoantibodies**

C. C. Calkins,[1] S. V. Setzer,[1] J. M. Jennings,[1] S. Summers,[1] K. Tsunoda,[2] M. Amagai,[2] A. P. Kowalczyk[1]; [1]Dermatology, Emory University School of Medicine, Atlanta, GA, [2]Department of Dermatology, Keio University School of Medicine, Tokyo, Japan

Desmosomes are adhesive intercellular junctions prominent in the skin and heart. Loss of desmosome function is associated with severe congenital and acquired disorders characterized by tissue fragility. Pemphigus vulgaris is an autoimmune disorder in which antibodies are directed against the desmosomal adhesion molecule Dsg3, resulting in severe mucosal erosions and epidermal blistering. To define the mechanisms by which Dsg3 autoantibodies disrupt keratinocyte adhesion, the fate of PV IgG and various desmosomal components was monitored in primary human keratinocytes exposed to PV patient IgG. PV IgG initially bound to keratinocyte cell surfaces and colocalized with desmosomal markers. Within 6 hrs after PV IgG binding to Dsg3, electron microscopy revealed that desmosomes were dramatically disrupted and keratinocyte adhesion was severely compromised. Immunofluorescence analysis indicated that PV IgG and Dsg3 were rapidly internalized from the cell surface in a complex with plakoglobin, but not desmoplakin. Dsg3 internalization was associated with disrupted desmoplakin organization and retraction of keratin filaments from cell-cell borders. The internalized PV IgG-Dsg3 complex colocalized with markers for both early and late endosomes, suggesting that Dsg3 was targeted for degradation. Consistent with this possibility, biotinylation experiments demonstrated that soluble Dsg3 cell surface pools were rapidly depleted, followed by loss of insoluble Dsg3. These findings demonstrate that the loss of keratinocyte adhesion in cells exposed to PV IgG is coupled to Dsg3 endocytosis and degradation.

1858

**Myogenesis Induction is Regulated by Association of N-cadherin with Lipid Rafts**

N. Taulet, F. Comunale, C. Gauthier-Rouvière; Cell Biology, CRBM - CNRS FRE 2593, Montpellier, France

Cell-cell adhesion mediated by cadherins is involved in a wide variety of cellular functions including cell growth, migration and differentiation. These transmembrane glycoproteins act as adhesive receptors when they accumulate at cell-cell contact sites where they promote intracellular signaling pathways. Our study focuses on N-cadherin which plays an important role for myogenesis induction through the positive regulation of RhoA and the negative regulation of Rac1. We have recently shown, in C2C12 myoblasts, that N-cadherin and catenins (α, β, γ and p120) association with membrane microdomains enriched in cholesterol called lipid rafts participates in adhesive complex stabilization (Causeret M./Taulet N. *Mol. Biol. Cell* 2005). Moreover, our results indicate that lipid rafts disruption leads to disorganization of N-cadherin dependent cell-cell contacts and inhibition of myogenesis, suggesting that this association is necessary to promote skeletal muscle differentiation. In the present study, we investigate how the presence of N-cadherin and catenins in lipid rafts might control myogenesis induction through the regulation of Rho GTPases activity, especially RhoA activation. In order to identify the signaling pathways implicated in this process, we try to highlight the dynamic assembly of signaling proteins with N-cadherin in lipid rafts.

1859

**Advanced-glycation End-products Increase Vascular Permeability *In Vitro* through Increased Protease Activity**

D. Navaratna; Cell Biology and Physiology, School of Medicine, University of New Mexico, Albuquerque, NM

Increased vascular permeability is one of the foremost microvascular complications in early non-proliferative diabetic retinopathy. Recently, several studies have implicated advanced glycation end-products (AGEs) and their downstream signaling molecules as significant contributors to increased microvascular permeability and endothelial cell dysfunction in the retina. However, the mechanistic basis of this AGE-induced permeability remains unexplored. The aim of this study was to determine if AGE-BSA alters the cellular distribution of VE-cadherin in endothelial lateral junctions and if this alteration is mediated through increased protease activity. Extracellular proteinases regulate cellular function at various levels and have been shown to play a pivotal role in disrupting the blood-brain barrier. Treatment of bovine retinal microvascular endothelial cells with AGE-BSA, an accepted prototype of advanced glycation end-products led to altered cellular morphology, disruption of lateral junctions and a reduction of VE-cadherin expression on the cell surface. This was accompanied by a 2-fold increase in permeability upon AGE stimulation in an in vitro FITC-dextran permeability assay. Inhibitors of protease activity were able to successfully abolish the action of AGE-BSA on VE-cadherin expression and restore permeability back to near control levels. A separate in vitro experiment showed that MMP-9 is able to cleave VE-cadherin in cell extracts and on the surface of intact cells possibly leading to subsequent internalization. Our findings point out to a novel role for proteases in altering endothelial barrier function that might dictate effective strategies for therapeutic intervention in vivo.

1860

**Prototypical Type-I E-cadherin and Type-II Cadherin-7 Mediate Very Different Distinct Adhesiveness through their Extracellular Domain**

Y. Chu,[1] O. Eder,[1] W. A. Thomas,[2] I. Simcha,[3] A. Ze'ev,[3] F. Pincet,[4] J. Thiery,[1] S. Dufour[1]; [1]Umr144 cnrs, Institut Curie, Paris, France, [2]Natural Sciences, Colby-Sawyer College, New London, NH, [3]Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel, [4]Umr8550 cnrs, ENS, Paris, France

Using a dual pipette assay that measures the force required to separate adherent cell doublets, we have quantitatively compared intercellular adhesiveness mediated by Type-I (E- or N-cadherin) or Type-II (cadherin-7 or -11) cadherins. At similar cadherin expression levels, cells expressing Type-I cadherins adhered much more rapidly and strongly than cells expressing Type-II cadherins. Using chimeric cadherins, we found that the extracellular domain exerts by far the dominant effect on cell adhesivity, that of E-cadherin conferring high adhesivity, that of cadherin-7 conferring low adhesivity. Type-I cadherins were incorporated to a greater extent into detergent-insoluble cytoskeletal complexes, and their cytoplasmic tails were much more effective in disrupting strong adherent junctions, suggesting that Type-II cadherins form less stable complexes with β-catenin. The present study demonstrates compellingly, for the first time, that cadherins are dramatically different in their ability to promote intercellular adhesiveness, a finding that has profound implications for the regulation of tissue morphogenesis.

## Gap Junctions (1861-1884)

1861

**Interplay Between CFTR and Cx45, Cx32 and Cx40, but not Cx50, Channels**

B. A. Kotsias, M. Salim, L. L. Peracchia, C. Peracchia; Pharmacology and Physiology, University of Rochester, Rochester, NY

The cystic fibrosis transmembrane regulator (CFTR) influences the function of other channels via unknown mechanisms. Chanson et al. (1999) have shown that CFTR activation increases the $Cl^-$ current and gap junctional conductance (Gj) of pancreatic cells expressing Cx45. We have confirmed these data in *Xenopus* oocyte pairs coexpressing CFTR and Cx45; in addition, we have reported a decrease in Vj sensitivity, demonstrated by an increase in $G_{j\ steady\ state}/G_{j\ peak}$ at the pulse (Kotsias and Peracchia, 2005). Recently, we have obtained similar results with Cx32 and Cx40, but not Cx50. Application of 20-40 μM forskolin to oocyte pairs clamped at -20 or -40mV and subjected to -40 or -60mV Vj pulses (12s duration) induced a $Cl^-$ current, increased $G_{j\ peak}$ by 30±6% (n=5) and 600±240% (n=6) in Cx32 and Cx40, respectively, and increased $G_{j\ steady\ state}/G_{j\ peak}$ by 28% and 55%, respectively. In oocyte pairs expressing just Cx40 (or Cx45) in one oocyte (#1) and both Cx40 (or Cx45) and CFTR in the other (#2), with negative pulses applied to oocyte #1 forskolin application still increased Gj and decreased Vj sensitivity. $G_{j\ peak}$ increased by 143±25% (n=3) and 61±19% (n=7) in Cx40 and Cx45, respectively, and increased $G_{j\ steady\ state}/G_{j\ peak}$ by 96% and 50%, respectively. This indicates that CFTR activation is effective even when it affects only one of the two hemichannels and that the Gj and Vj changes are not artifacts of decreased membrane resistance in the pulsed oocyte. Preliminary data show that in oocytes coexpressing CFTR and Cx40-TR (Cx40 with COOH-terminus truncated beyond residue 248) forskolin has no effect on Gj and Vj sensitivity. This suggests that the COOH-terminus plays a role in the interplay between CFTR and Cx40 channels. Supported by NIH grant GM20113.

1862

**E-Cadherin Differentially Regulates the Fate of Connexin43 and Connexin32 in A431D Cells**

S. Chakraborty, S. Mitra, R. Govindarajan, K. Knudtson, P. P. Mehta; Biochemistry and Molecular Biology, University of Nebraska Medical

Center, Omaha, NE
It is as yet unknown how cadherin-mediated cell-cell adhesion facilitates the assembly of connexin(Cx)s into gap junction(GJ)s. Because the C-terminal tail of Cxs varies significantly in amino acid identity, we hypothesized that assembly of Cxs into GJs is regulated by C-terminal tail of Cxs and their interaction with the cadherins, or both. We used cadherin-null, human epidermoid carcinoma-derived A431D cells, and an alpha-type (Cx43) and a beta-type (Cx32) Cx that have divergent C-terminal tails, to test our hypothesis. Immunocytochemical analysis following transient expression of these Cxs revealed that both Cxs failed to assemble into GJs and accumulated intracellularly in the Golgi and early endosomal compartment. Transient expression of both Cxs along with E-cadherin in A431D cells induced the assembly of Cx43, but not Cx32, into GJs. Significant colocalization of Cx43 and E-cadherin was observed not only at the cell-cell contact areas but also in the sub-cellular compartments. These observations were substantiated by transiently expressing Cx43 and Cx32 in A431DE cells that were engineered to express E-cadherin stably. To investigate if the C-terminal tail of Cxs regulates their subcellular fate in the presence of E-cadherin, we transiently expressed A431DE cells with C-terminal tail deleted mutants of Cx43 and Cx32. We found that the mutant Cxs accumulated intracellularly and did not assemble into GJs. These data suggest that the C-terminal tail of Cx43 harbors motif(s) that allows it to interact with E-cadherin, either directly or indirectly, and assemble into GJs. These findings suggest that the trafficking and assembly of Cxs are regulated by domains in the Cxs that are Cx subtype dependent and by the interaction among Cxs and cadherins, or both.(Supported by NIH CA113901)

1863

**Cell Adhesion Proteins and Gap Junction Formation**

M. A. Matrone,[1] D. M. Abel,[1] S. Braconnot,[2] M. M. Falk[1]; [1]Biological Sciences, Lehigh University, Bethlehem, PA, [2]Macromolecular Systems & Human Physiology, CNRS-bioMérieux, ENS de Lyon, Lyon, France

In the plasma membrane, gap junction channels typically cluster to provide direct cell-cell communication between adjacent cells. Previous studies suggest that “helper” proteins, such as cadherins and claudins found in adherens and tights junctions, respectively, are necessary for connexons to dock into double-membrane-spanning gap junction channels, perhaps by reducing the natural distance of adjacent plasma membranes to within a certain threshold. To identify where the first connexons dock, high-resolution immunofluorescence microscopy in T51B cells, which endogenously express Cx43, demonstrated that the first gap junction plaques were formed at adherens junction-mediated cell-cell adhesion sites, where the distance between the adjacent cell membranes is reduced. Abolishing adherens junction formation at the plasma membrane by calcium-depletion revealed the reversible abolition of gap junction formation. Return of calcium to the growth media allowed for the reformation of adherens junctions, followed quickly by the reformation of gap junctions. Similarly, use of a dominant negative N-cadherin mutant, which lacks the N-terminal calcium-binding domains, dramatically reduces adherens junction formation by depleting the available catenin pool, and, thus, reduces gap junction formation. Provided that the loss of adherens junctions observed in these experiments results in markedly reduced or abolished gap junction formation, it remains uncertain whether the membrane proximity conferred by cell adhesion proteins affects connexon docking/clustering and/or connexin expression/localization. To investigate this, genetically manipulated cadherin proteins were engineered and characterized in regard to their effect on gap junction formation.

1864

**Intracellular Site of Gap Junction Protein Oligomerization**

L. Musil, J. VanSlyke; Oregon Health and Science U., Portland, OR

Oligomerization of connexin molecules into hexameric connexons (hemichannels) is a key step in gap junction assembly. Using five mechanistically distinct blockers of specific steps in intracellular transport including brefeldin A (BFA), Musil and Goodenough (1993) demonstrated that endogenously expressed, $^{35}$S-methionine metabolically labeled Cx43 assembles into connexons in a post-endoplasmic reticulum (ER), pre-plasma membrane compartment likely to be the trans Golgi network (TGN). It is not known whether this unusual site of assembly is a general property of connexins, or (as has recently been proposed) is restricted to Cx43 and other alpha group members. To address this issue, we used our previously established chemical cross-linking techniques to examine the assembly of Cx32 in two cell types that naturally express this beta-type connexin. In both cases, oligomerization of newly synthesized $^{35}$S-met-Cx32 into connexons was abolished when transport out of the ER was blocked by brefeldin A (BFA). In contrast, PC12 and HeLa cells stably transfected with plasmids encoding WT Cx32 driven by viral promoters oligomerized $^{35}$Smet-Cx32 into connexons even in the presence of BFA. Similar results were obtained with Cx43 in C6 cells: although assembly of endogenous Cx43 into connexons occurred exclusively after ER exit (e.g., was BFA-sensitive), BFA did not block connexon formation in transfectants expressing high levels of exogenous Cx43. Moreover, a cell line that simultaneously synthesizes both endogenous Cx43 and exogenous Cx32 oligomerized only the latter into hemichannels under conditions that prevented ER exit. We conclude that natively expressed Cx32, like Cx43, is assembled into connexons only after its transport out of the ER. High-level overexpression of either connexin can induce premature oligomerization within the ER, which may indicate that connexon formation is largely a self-assembly process driven by the local abundance of connexin monomers.

1865

**Gap Junction Degradation in Clathrin and Dab2 Depleted Cells**

A. Gumpert, M. Falk; Biological Sciences, Lehigh University, Bethlehem, PA

Gap Junction channels consist of two single-membrane spanning half-channels, which provide direct cell-to-cell communication between adjoining cells. Once connected the two half-channels cannot be separated under physiological conditions. It has been shown that the half-life of Gap Junction proteins is only a few hours and that the double-membrane channel clusters are degraded via internalization of the cluster into one of the two coupled cells. The internalization of gap junction cluster or its portion is believed to lead to the formation of a double-membrane vesicle termed “annular junction” Experiments indicate that the internalization of gap junction employs proteins like clathrin, AP-2 and Dab2 which are know to participate in receptor mediated endocytosis. To show the proteins’ involvement in gap junction degradation we investigate the internalization of gap junction clusters after down-regulation of proteins known to be essential in endocytosis and by site directed mutagenesis. The internalization process is studied in clathrin, AP-2 and Dab2 depleted cells. Down-regulation of protein expression, achieved by transient transfections with short interfering RNAs, and gap junction protein internalization are demonstrated by Western blotting and by

immunoflourescence staining respectively.

1866

**Double-Membrane Gap Junction Vesicles Generated by a Clathrin-Mediated Endocytosis-Like Mechanism**

M. Piehl,[1] C. Lehmann,[1] D. Segretain,[2] M. Falk[1]; [1]Biological Sciences, Lehigh University, Bethlehem, PA, [2]INSERM U670, Universite de Paris 5, Paris, France

To provide direct cell-to-cell communication, gap junction (GJ) channels connect the cytoplasm of two adjacent cells by traversing the apposing plasma membranes. These GJ channels (one half-channel from each cell) cluster into large GJ plaques (up to several square µms in diameter), and cannot be split under physiological conditions. Although gating can regulate GJ channels, biochemical analyses have shown that GJ proteins are turned over quite rapidly (half-life = 2-3 hours). This suggests that cells need to degrade GJ plaques efficiently. The degradation process may occur via the unusual invagination of double-membrane GJ plaques into one of the two coupled cells, consequently forming double-membrane vesicles termed annular gap junctions (AGJs). These AGJs have been previously described by ultra-structural analysis of differentiating tissues and cells in culture. Here we report that entire GJ plaques, or large portions of plaques, are internalized to form double-membrane AGJs (one membrane from each cell). These AGJs are then fragmented into smaller vesicles that are degraded preferentially by phago-lysosomal pathways. Surprisingly, functional analysis of components involved in GJ plaque internalization revealed an endocytosis-like mechanism. This mechanism involves the coat protein clathrin, the alternative adaptor protein Dab2, dynamin, myosin-VI, and actin filaments. Our results indicate that these components are responsible for the internalization, inward movement, and degradation of GJ plaques. To our knowledge, this is the first time receptor-mediated endocytosis components were shown to be involved in the generation of double-membrane vesicles.

1867

**Connexin 43 Degradation is Associated with its Aberrant Localization in Lung Tumor Cells**

S. C. Pfister,[1] R. V. Santelli,[2] G. M. M. Santelli[1]; [1]Biologia Celular e do Desenvolvimento, University of São Paulo, São Paulo, Brazil, [2]Biochemistry, University of São Paulo, São Paulo, Brazil

Gap Junction Channels (GJCs), structures present in plasma membrane of contacting cells, have been associated with tumor suppression. Gap Junctions (GJs) are composed by connexons, GJC precursors, require hexameric proteins named connexins. Even though the connexin family consists of around 20 members, only few isoforms are detected on distinct cells. Abnormal GJC may be consequence of failures in assembling or connexin degradation. So, the goal of this study was to identify if the degradation routes of connexin 43 has influenced the GJC maintenance. First, we compared some GJC features between normal hepatocyte cells (BRL3A) and hepatocarcinomas (HTC), both derived from *Rattus norvegicus.* Immunofluorescence reactions and Lucifer Yellow assays showed significant formation of functional channels in BRL3A while HTC cells presented not-functional GJC and connexin 43 was modestly detected on intracellular compartments. In a second step, HTC cells transfected with connexin 43 fused to Green Fluorescent Protein (Cx43-GFP) have restored GJC formation. Furthermore, HTC expressing Cx43-GFP reduced significantly cell growth when compared to HTC cells. Analysis by immunoblotting permitted us to identify that Epidemical Growth Factor Receptor (EGFR) was also decreased in HTC expressing Cx43-GFP. To identify if failures on cell trafficking is responsible to abnormal characteristics of GJCs, we used some inhibitors of both lysosomes and proteasoma pathways, besides a treatment with a drug that disrupts the Golgi transport, Brefeldin A. The present data suggest that abnormalities in degradation of connexin 43 are associated with GJC of lung tumor cells.

1868

**3D Microscopic Localization of Connexin43 Phospho-specific Isotypes**

G. M. Hand,[1] G. M. Gaietta,[1] B. N. Giepmans,[1] J. L. Solan,[2] P. D. Lampe,[2] G. E. Sosinsky[1]; [1]National Center for Microscopy and Imaging Research, Dept. of Neurosciences, University of California, San Diego, La Jolla, CA, [2]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA

Cell-cell communication via gap junctions is dependent on the assembly, transport and formation of cell-to-cell channels formed by connexin (Cx) proteins. Multiple phosphorylation sites are present in the abundant and best-studied connexin isoform, Cx43. Phosphorylation is involved in proper protein trafficking, assembly, degradation and regulation of channel gating. Our goal is to determine the sub-cellular localization of Cx43 specific phospho-forms. We use a combination of immunolabeling, tyramide signal amplification (TSA), fluorescence photooxidation and electron microscopic tomography (EMT) to study the distribution of different Cx43 phospho-types. Cell lysates from non-stimulated NRK cells contain multiple Cx43 isoforms, including a non-phosphorylated form (P0 or NP form, although additional analysis indicates phosphorylated and non-phosphorylated Cx43 species can co-migrate) and two phosphorylated forms (P1 and P2). P1 and P2 are phosphorylated on multiple, unidentified serine sites and are the main form in native gap junction plaques. In immunostainings performed with Zymed monoclonal antibody 13-8300, we found strong intracellular, perinuclear fluorescence and far less gap junctional staining. This antibody labels only the P0 form of Cx43 (Nagy et al. (1997) Exp. Cell Res. 236:127-136). Subsequently, we used a different monoclonal antibody (CT antibody) that preferentially stains the P0 form as well as an antibody that stains primarily gap junction plaques (IF antibody). CT and IF antibodies were both made against the same peptide, located between amino acids 360-382 in the Cx43 sequence. Current studies are testing the hypothesis that the CT antibody most likely includes S372 as part of its binding site and when S372 is phosphorylated the CT antibody no longer binds. Initial EMT results indicate that the P0 form of Cx43 is primarily localized in the lumen of Golgi tubules. Analysis of tomograms provides a 3D representation of this labeling and its relationship to other Golgi components.

1869

**Truncation of the Gap Junction Protein Cx43 Changes the Molecular Composition of the Intercalated Disc**

K. Maass,[1] J. Shibayama,[1] S. E. Chase,[1] S. F. Noujaim,[1] K. Willecke,[2] M. Delmar[1]; [1]Pharmacology, SUNY Upstate Medical University, Syracuse, NY, [2]Institut fuer Genetik, Universitaet Bonn, Bonn, Germany

OBJECTIVE: To analyze the effect of the Cx43K258stop mutation on intercalated disc organization and on cardiac function in mice. INTRODUCTION: Recent studies indicate that the carboxy terminal (CT) region of connexin43 (Cx43) acts as a regulatory domain in the gating of

gap junction channels and is also involved in the direct interaction with other proteins. To analyze the function of the Cx43CT *in vivo*, mice expressing a mutant isoform lacking most of the Cx43CT (Cx43K258stop) in place of Cx43 were recently generated. Homozygous Cx43K258stop mice died postnatally due to a defective epidermal barrier; however, most mice harbouring only one *Cx43K258stop* and one *cx43 knockout* allele (Cx43K258stop/Cx43KO) survived to adulthood. Hence, hearts of Cx43K258stop/Cx43KO animals were used for the experiments described below. RESULTS: Immunolocalization studies on wild type adult cardiac ventricles showed that, consistent with previous studies, the position of Cx43 signals at the intercalated disc alternated with that of adherens junction proteins such as N-cadherin, p120-, alpha- and beta-catenin. In contrast, the Cx43K258stop protein segregated from adherens junctions, forming enlarged plaques at the lateral boundaries of the intercalated disc. Similar changes in co-localization were detected for the proteins ZO-1 and gamma-catenin. Separate studies are in progress to determine whether the Cx43K258stop mutation has an effect on cardiac function, particularly regarding the electrophysiological properties under normal and ischemic conditions. CONCLUSION: In the adult murine heart, lack of the Cx43CT is associated with larger gap junction plaques and segregation of Cx43 from other junctional proteins at the intercalated disc. The functional consequences of this molecular rearrangement remain to be determined.

1870

**Production of a 20kD Fragment by Intramembrane Proteolysis of Connexin43**

R. Joshi-Mukherjee,[1] C. Burrer,[2] L. Gao,[1] W. Coombs,[1] M. Delmar,[1] S. Taffet[2]; [1]Pharmacology, SUNY Upstate Medical Univeristy, Syracuse, NY, [2]Microbiology and Immunology, SUNY Upstate Medical Univeristy, Syracuse, NY

In vertebrates, gap junctions are formed by oligomerization of a protein subunit called connexin. One of the most prevalent connexin isotypes is connexin43 (Cx43). This protein is immunodetected as a 43kD band. In addition, in some cell lines, Cx43 antibodies directed to the carboxyl terminal domain allow for the detection of a ~20kD band. Previous studies suggested that the ~20kD fragment may result from protein degradation during sample handling. However, here we show that protection of the sample using a commercial protease inhibitor (Roche Complete®) along with various levels of PMSF (1-5 mM) did not prevent the production of this ~20kD fragment. This indicates that the fragment is not the result of degradation during sample preparation but the result of processing within the cell. Given the fragment size (and assuming that the fragment extends to the very C-end of the Cx43 sequence) we estimated that the point of proteolysis would be localized within the 4$^{th}$ transmembrane domain. Interestingly, the sequence of this domain presents a consensus site for intramembrane proteolysis by signal peptidase. Hence, the potential cleavage site L228-F229 was replaced with K228-K229. The construct was transfected into N2a cells. Consequent to the mutation, Cx43-CT antibodies failed to detect the 20kD fragment. As a separate approach to assess *in vivo* Cx43 processing, we induced the fragment in a cell line where it would be normally undetectable. CHO cells were used for this purpose. Exposure to TPA (a treatment known to activate intramembrane proteolysis) led to the production of the ~20kD fragment. Overall, our data strongly support the notion that the ~20kD fragment is a result of intramembrane proteolysis due to the constitutive expression of an intramembrane protease or, alternatively, by a mechanism that is induced by TPA treatment.

1871

**Aminosulfonate Inhibition of Cx26-containing Channels Involves the C-terminal Domain**

l. Tao, A. L. Harris; Pharmacology/physiology, UMDNJ, Newark, NJ

The protonated form of taurine and other aminosulfonates directly inhibited heteromeric connexin 26/connexin32 (Cx26/Cx32) hemichannels purified from rodent liver and reconstituted into liposomes (JBC 274:3711& 279:38544). The mechanism of the inhibition was examined in reconstituted hemichannels and HeLa junctional channels composed Cx26, Cx32 or both, with and without a cleavable C-terminal tag (T) consisting of an hemagglutinin-6x(HN) epitope (Biochem J. 383:111). Hemichannel inhibition was assessed by transport-specific fractionation of liposomes (TSF). In contrast to Cx26/Cx32 channels, Cx26T/Cx32 and Cx26T hemichannels were not inhibited by aminosulnate. On the other hand, Cx26/Cx32T hemichannels were sensitive. This suggested that Cx26 tag blocked the aminosulnate sensitivity. To explore this possibility, effects of tag cleavage were examined. Inhibition was restored when the tag was cleaved from Cx26T/Cx32 channels (Cx26Tc/Cx32). Cx26/Cx32Tc hemichannels remained sensitive, but, surprisingly, Cx26Tc hemichannels remained insensitive. These results confirmed that the Cx26 C-terminal tag interferes with aminosulfonate sensitivity, and suggest that the sensitivity requires Cx32 in the same channels. Hemichannel results were corroborated by studies of junctional channels, for which inhibition was assessed by the “parachute” dye-coupling assay (Bio. Tech. 18:490). Extracellular taurine causes cytoplasmic taurine levels to increase, due to the endogenous taurine transport in HeLa cells. Taurine substantially reduced dye coupling of junctional channels composed of Cx26/Cx32T, but not Cx26T, Cx26T/Cx32 or Cx32T. Taurine-induced inhibition was blocked by presence of HEPES in the medium, which blocks the taurine transport and is not membrane-permeable. Thus the junctional channel results corresponding reconstituted hemichannels. These data show that aminosulfunote inhibition is blocked by a Cx26 C-terminal-tag, that Cx32 is required and that junctional channels have the sensitivity of the corresponding reconstituted hemichannels. (Supported by NIH GM36044 and GM61406)

1872

**Identification of Residues of the Third Transmembrane Helix of Connexin 43 (Cx43) that Line the Gap-Junctional Hemichannel (GJH) Pore**

S. Lee, L. Reuss, G. A. Altenberg; Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX

The gap-junctional channels that mediate intercellular communication are formed by head-to-head docking of two connexons or gap-junctional hemichannels (GJH) from adjacent cells. GJH are connexin (Cx) hexamers and each Cx has four transmembrane domains (M1-M4). A definitive identification of the transmembrane helices that line the gap-junctional pore is not available. Here, we used a sensitive technique (the uptake of carboxyfluorescein, CF, elicited by lowering extracellular [$Ca^{2+}$] by GJH in frog oocytes) to identify residues in M3 that face the pore of Cx43 GJH. Cx43 is expressed in several tissues and organs, including brain, myocardium, kidney and capillary endothelial cells. We have previously demonstrated that the uptake of CF in oocytes injected with Cx43 cRNA is *via* GJH and that Cys-less version of Cx43 forms functional GJH. We substituted individually each M3 native residue with Cys, and then determine where reaction with the cell-membrane impermeable thiol-selective biotinylation reagent MBB ($M_r$ 537) affected CF uptake. All mutants showed CF uptake upon lowering [$Ca^{2+}$], but this CF uptake was sensitive to the inhibition by MBB only in the mutants I156C, F161C, V164C and V167C (-74 ± 5 %, mean ± SEM, $P < 0.001$). Prior reaction with the small

hydrophilic thiol reagent iodoacetic acid ($M_r$ 186) prevented the block by MBB in F161C, V164C and V167C. These results indicate that none of the native M3 residues is essential for GJH assembly and function, and suggest a face of the M3 helix lining the Cx43 pore that is consistent with a recent structure model of gap-junctional channels.

1873

**Functional Comparison of Connexin 26 Wild Type and Deafness Causing Mutant Channels**

G. Mese,[1] V. Valiunas,[2] P. R. Brink,[2] T. W. White[2,1]; [1]Graduate Program in Genetics, SUNY at Stony Brook, Stony Brook, NY, [2]Department of Physiology and Biophysics, SUNY at Stony Brook, Stony Brook, NY

There are at least four connexins (Cxs) in the cochlea and mutations in Cx26, Cx30 and Cx31 are leading causes of hereditary deafness. Differential selectivity of connexins to different molecules and second messengers is one basis of their uniqueness, therefore compensation for the absence of even a single connexin is challenging in the inner ear. Contrasting the permeability of human Cx26 deafness associated mutations with wild type Cx26 may provide insight for characterizing the molecules that need to be shared between cells for normal cochlear function. For this study, two human Cx26 mutations which retained some level of activity, Thr8Met (T8M) and Asn206Ser (N206S), were investigated. In order to assess the differences in the single channel properties and permselectivity, wild type and mutant Cx26 constructs were transfected into connexin deficient mouse neuroblastoma cell lines (N2A). Individual cell pairs were analyzed by dual whole-cell voltage clamp and intercellular fluorescent dye transfer. The average unitary conductance of wild type channels was 107±17pS using a pipette solution with 120mM K-aspartate which facilitated the examination of channel permeability to potassium ions. Similarly, N206S and T8M mutants had unitary conductances of 105±20pS and 106±19pS, respectively. These observations indicated that N206S and T8M mutant channels were as permeable to potassium ions as wild type Cx26 junctions. The permeability of mutant and wild-type channels to Lucifer yellow (LY) were also analyzed in individual cell pairs. The initial observations suggest that LY transfer through the mutant channels was also comparable to that of wild-type channels. Further investigation of Cx26 wild type and mutant channels may identify a difference in their permselectivity, providing a basic understanding about the etiology of the hereditary deafness in molecular level.

1874

**Connexin 43 Hemichannels: Oxidative Stress Increases Open Probability**

M. V. L. Bennett,[1] M. A. Retamal,[2] C. Cortés,[2] L. Reuss,[3] J. C. Saez[2]; [1]Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, [2]Departamento de Ciencias Fisiológicas, Pontificia Universidad Católica de Chile, Santiago, Chile, [3]Sealy Center for Structural Biology and Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX

Cx43 hemichannels are formed in intracellular membranes and transported in vesicles that are inserted into the surface membrane. They diffuse in the surface to dock with hemichannels in an apposed membrane to form the full cell-cell channels. The surface but nonjunctional hemichannels open rarely, but opening is increased by metabolic inhibition (MI) with iodoacetate and antimycin A ("chemical ischemia"). Opening is detected by electrophysiological measurement of single channel conductances about twice that of the cell-cell channels and by dye uptake that is prevented by gap junction blockers and restricted to molecules that permeate gap junctions. Dephosphorylation and/or oxidation of Cx43 were proposed as potential mechanisms of hemichannel opening, since MI leads to dephosphorylation of total Cx43 and reducing agents can prevent dye uptake. Here using rat cortical astrocytes in primary culture, we isolated surface proteins by biotinylation followed by Western blotting. MI about doubled the amount of Cx43 on the cell surface in 15 min with a small further increase by 50 min. Dephosphorylation began at about 15 min MI and was nearly complete by 50 min. S-nitrosylation of Cx43 was detected by 30-40 min. DTT, a membrane permeant reducing agent, did not affect dephosphorylation but almost completely blocked hemichannel opening and S-nitrosylation. Hemichannel opening was also blocked by glutathione ethyl ester, which is membrane permeant, but not by glutathione, which is membrane impermeant. Moreover, GSNO, a membrane permeant nitric oxide donor, induced dye uptake and Cx43 nitrosylation, but did not affect the levels of Cx43 in the surface or its phosphorylation state. We propose that S-nitrosylation of intracellular Cx43 cysteine residues is a critical factor in the opening of Cx43 hemichannels induced by MI, a chemical treatment mimicking ischemia.

1875

**Connexin47 (Cx47) and Cx32 in Adult Rodent CNS: A Major Role for Oligodendrocyte Gap junctions in "Dynamic Potassium Siphoning"**

T. Yasumura,[1] N. Kamasawa,[1] K. G. V. Davidson,[1] A. Sik,[2] J. I. Nagy,[3] J. E. Rash[1]; [1]Biomedical Sciences, Colorado State University, Fort Collins, CO, [2]Centre de Recherche, University Laval Robert-Giffard, Quebec, PQ, Canada, [3]Physiology, University of Manitoba, Winnipeg, MB, Canada

Cx47/Cx32 double-knockout mice have severe seizures, the innermost layer of their myelin sheaths is grossly swollen, and all mice die by 10 weeks of age (Odermatt et al., 2003). We analyzed the subcellular distributions of Cx47 and Cx32 in normal adult rodent CNS. By confocal microscopy, Cx47 was co-localized with astrocytic Cx43 on oligodendrocyte somata, and along myelinated fibers, whereas Cx32 without Cx47 was co-localized with contactin-associated protein (caspr) in paranodes. By freeze-fracture replica immunogold labeling, large gap junctions between oligodendrocyte somata and astrocyte processes contained much more Cx47 than Cx32. Along surfaces of internodal myelin, Cx47 was several times as abundant as Cx32 and often occurred without Cx32. In contrast, Cx32 was localized without Cx47 in autologous gap junctions in Schmidt-Lanterman incisures and between paranodal loops bordering nodes of Ranvier. Thus, Cx47 is the most abundant connexin in heterologous oligodendrocyte-to-astrocyte gap junctions, whereas Cx32 is the predominant if not sole connexin in autologous ("reflexive") oligodendrocyte gap junctions. Autologous gap junctions between myelin layers and heterologous gap junctions linking oligodendrocytes to the astrocyte syncytium are proposed to act in electrical series to facilitate long-distance intercellular "dynamic $K^+$ siphoning". $K^+$ at high concentration in the depolarized (+40 mV) innermost layer of myelin is proposed to flow down its electrochemical gradient, through paranodal and Schmidt-Lanterman gap junctions, to the outermost layer of myelin (−70mv), thence through oligodendrocyte/astrocyte gap junctions to the astrocyte endfeet (−85 mV) that form the glia limitans (total 125mV potential difference). Elimination of all oligodendrocyte gap junction pathways for $K^+$ siphoning in Cx32+Cx47 double-knockout mice would account for $K^+$ accumulation and extensive swelling of myelin in these animals. Supported by NIH NS44010 and NS44395 (JER) and CIHR (JIN and AS)

1876

**Transient Opening of Connexin 32 Hemichannels in Liver Releases ATP and Functions as an Early Signal for the Onset of Regeneration after Partial Hepatectomy in Rats**

S. Crumm, J. B. Hoek; Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philladelphia, PA

Liver regeneration after partial hepatectomy (PHx) is orchestrated by a variety of signals from cytokines and growth factors. However, the onset of regeneration remains poorly understood. Here, we report a marked (30%) and rapid (30sec) decrease in ATP, total adenine nucleotides (TAN) and $NAD^+$ levels in the remnant liver following PHx, resulting in a change in ATP/AMP ratio. These changes were followed by activation of AMP-activated protein kinase (AMPK) with phosphorylation of both α1 and α2 isoforms of AMPK. Changes in TAN were not due to increased energy demand and pretreatment with L-NAME (NOS inhibitor) or phentolamine (α1-adrenergic receptor antagonist), which have been previously reported to inhibit liver regeneration, prevented the loss of TAN and $NAD^+$ and inhibited AMPK activation, suggesting a role for an early stress response in the liver. The loss of TAN and $NAD^+$ was also prevented by treatment with 18-α-glycerrhetinic acid, an inhibitor of gap junctions, or with peptide inhibitors against connexin 32 (32-GAP26 and 32-GAP27), which block connexin 32 hemichannels by binding the extracellular connexin 32 interaction domain. The connexin 32 inhibitors also prevented phosphorylation of AMPK α subunits in the remnant liver. By contrast, peptide inhibitors against connexin 43 were only partially effective. These findings indicate that PHX causes a stress response in the remnant liver that induces a transient opening of connexin 32 hemichannels in hepatocytes resulting in a rapid release of ATP and $NAD^+$ and activation of AMPK. We suggest that these effects constitute an early signal for the onset of liver regeneration after PHx.

1877

**Shear Stress Alters Connexin Expression and Gap Junction Communication in Endothelial Cells**

T. L. Johnson, R. M. Nerem; Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA

Vascular gap junctions maintain vessel function by allowing the passage of vasoactive molecules and second messengers within the endothelium and underlying smooth muscle cells. Altered expression of endothelial gap junction proteins (Connexins 37, 40 and 43) has been linked to atherosclerosis and hypertension; however their response to mechanical forces remains unclear. The goal of this study is to examine the influence of shear stress on endothelial connexin gene expression, protein expression and localization, and gap junction communication. Human aortic endothelial cells were cultured in monolayer on collagen I-coated glass slides and exposed to static or laminar shear stress (15 dynes/cm2) using a parallel plate flow chamber. Real time RT-PCR was used to quantify gene expression and flow cytometry was used to quantify protein expression. Immunohistochemistry was used to localize protein expression and transfer of biocytin after scrape loading was used to measure gap junction communication within the endothelial monolayer. Results show a differential effect of shear stress on connexin gene expression with a decrease in Cx37, increase in Cx40 and no change in Cx43. There is no significant difference in Cx37, Cx40 or Cx43 protein expression in shear and static samples however image analysis identifies connexin proteins at the cell membrane and in the cytoplasm. Dye transfer results show a significant reduction in gap junction communication following shear exposure with a dye coupling index of one recipient cell per donor cell in shear samples and five recipients per donor in static samples. We conclude that shear stress influences endothelial cell connexin expression and gap junction communication which has implications in vascular health and disease.

1878

**Role of ILK and Akt in the Transcriptional Regulation of Connexin32 in Rat Hepatocytes**

I. Plante, M. Charbonneau, D. G. Cyr; INRS-Institut Armand-Frappier, Université du Québec, Pointe-Claire, PQ, Canada

Gap junctions mediate intercellular communication through channels composed of proteins, termed connexins (Cxs). We have shown that Cx32, the principal hepatic Cx, is down-regulated in the liver of female rats exposed to hexachlorobenzene (HCB), an epigenetic environmental carcinogen. This is concomitant to an activation of the Integrin-linked kinase (ILK) pathway, leading to the activation and nuclear translocation of Akt and the inactivation of GSK3β. Using an *in vitro* model, the aim of this study was to demonstrate the role of the ILK in Cx32 down-regulation pathway by assessing down-stream phosphorylation targets of ILK. In order to mimic the activation of ILK pathway, a rat hepatocyte cell line, $MH_1C_1$, was transiently transfected with an expression vector for ILK (ILK+ cells). These ILK-overexpressing cells showed a significantly lower Cx32 mRNA levels. In ILK+ cells, Akt was also activated and translocated into the nucleus. Using an constitutively-active Akt expression vector, we then observed that Akt+ cells showed a decrease in Cx32 mRNA levels. $MH_1C_1$ exposed to two specific inhibitors of GSK3β demonstrated that Cx32 is not regulated by GSK3β. Overall, these experiments demonstrate that Cx32 is down-regulated by the ILK pathway activation in rat hepatocytes. Moreover, data suggest an important mechanistic function of the nuclear translocation of Akt in the down-regulation Cx32. In the HCB-treated rat model, modulation of transcription factor of promoter regions of Cx32 were observed concomitantly with the nuclear translocation of Akt. was present into the nucleus. Thus, future experiments will looked at transcription factors in Akt overexpressing cells.

1879

**Rat Mast Cells Communicate with Fibroblasts via Gap Junction Communications Both in Vivo and in Vitro**

S. R. Au,[1] K. Au,[2] G. C. Saggers,[2] H. P. Ehrlich[2]; [1]University of California Davis, Davis, CA, [2]Department of Plastic Surgery, Hershey Medical Center, Hershey, PA

Mast Cells (MC) are suggested to promote fibrotic conditions such as hypertrophic scarring and keloid formation. Do gap junction intercellular communications (GJIC) between MCs and fibroblasts play a role in advancing fibrosis? GJIC between fibroblasts and MCs was demonstrated by the passage of fluorescent dye from preloaded rat MCs into fibroblasts both in vivo and in vitro. MCs were harvested from the peritoneal cavity of adult male rats and isolated with a density gradient. The MCs were treated with two dyes, the plasma membranes were stained red with DiI and the cytoplasms labeled green with Calcein-AM. These pretreated MCs were injected into 1. subcutaneous implanted polyvinyl alcohol (PVA) sponges in rats, 2. human fibroblasts in monolayer culture, and 3. added into co-cultured rat MC-fibroblast populated collagen lattices (FPCL) a model of wound contraction. Two weeks after implantation of the PVA sponges, pretreated rat MCs were injected into PVA sponge implants. Sponges were harvested 4 hrs later, frozen and cryosectioned. Fluorescent microscopy revealed red (DiI) and green (Calcein-AM) fluorescent stained MCs associated with fluorescent green fibroblasts, which were devoid of fluorescent red staining. *In vitro* the dye preloaded MCs passed Calcein-AM into human fibroblasts in monolayer culture. Green fluorescent fibroblasts were found in co-cultured rat MC-FPCLs. Co-cultured rat MC-FPCLs

showed a 35% enhancement of lattice contraction compared to FPCLs without added MC. It is clear that freshly isolated rat MCs form GJIC with fibroblasts in granulation tissue and tissue culture. FPCL contraction is an *in vitro* model of scar contracture, a common feature of hypertrophic scar. The inclusion of MCs in the casting of FPCLs enhanced FPCL contraction. These findings support the notion that MCs interactions with fibroblasts through GJIC escalate the severity of fibrosis.

1880

**Aldosterone Protection of MDCK Cells from Inhibition of Cell-to-Cell Communication by Ouabain**

R. M. Genga, M. L. S. Ledbetter; Biology, College of the Holy Cross, Worcester, MA

Cultured animal cells can exchange small molecules through gap junctions in their adjoining membranes. If the $Na^+$, $K^+$-ATPase of the cells is inhibited by the cardiac glycoside ouabain, cells lose $K^+$ and gain $Na^+$ at a rate determined by the corresponding channels and the chemiosmotic potential. This in turn leads to diminished junction-mediated cell communication, as assessed by transfer of Lucifer Yellow (457D) but not Texas Red-conjugated dextran (10kD ) from donor cells to neighboring recipients, estimated by quantitative image analysis. For many types of cells communication reaches a minimum after several hours, but is readily reversible. We are eager to understand the mechanism of this process. Using cultured Madin-Darby canine kidney (MDCK; ATCC CCL34) cells, we conducted careful measurements that confirmed the inhibition of communication by ouabain. Immunofluorescence of connexin43, the primary connexin in MDCK cells, also decreased at the cell surface, accumulating temporarily in a cytoplasmic compartment before disappearing altogether. Addition of amiloride, a blocker of the $Na^+$ channel, protected the cells from inhibition, implying that the pathway requires $Na^+$ elevation as a consequence of ouabain treatment. Addition of the mineralocorticoid hormone aldosterone also protects the cells from inhibition. The mechanism of this protection is not clear, but it seems to require the involvement of the aldosterone receptor (AR), since spironolactone and eplerenone, both AR antagonists, prevent rescue of communication by aldosterone. Whether the protection by activation of the AR involves direct stimulation of gap junction metabolism, or more likely, involves modulation of the ion homeostasis of the cells is not yet clear. We wish to thank the National Science Foundation (DUE-0308559) for support of this work, the Fortin Foundation for a Summer Research Fellowship to RMG, and Pfizer Pharmaceuticals for a sample of eplerenone.

1881

**Cell-Cell Cooperation Between Epithelial Cells: The Role of Hormone Ouabain**

I. Larre, A. Ponce, L. Shoshani, R. G. Contreras, A. Lázaro, M. Cereijido; Department of Physiology, Biophysics and Neurosciences, CINVESTAV, México, Mexico

We have previously found that ouabain-resistant MDCK cells (R-MDCK) can rescue sensitive ones in co-cultures, in the apparent absence of communicating junctions (Cereijido et al., 1985; Bolivar et al., 1987). On the basis that at that time ouabain was considered to be a toxin of vegetal origin, the problem was taken as a sort of pharmacological artifact and its elucidation was not pursued. Yet, since now ouabain is regarded as a hormone, whose function awaits to be elucidated, in the present work we reinvestigate the R/W cooperation. While studies with Lucifer Yellow fully confirm our previous data, Neurobiotin, a probe of smaller size and opposite electric charge (+ instead of -) that was not available in 1985-1987, shows that cells maintain sufficient communication to explain rescuing. Interestingly, the degree of communication is significantly enhanced ($p < 0.001$) by ouabain. However, an exchange of ions between R and W cells may not account for the observations reported below. Thus, in previous work we have found that ouabain triggers a P→A mechanism (from ***p***ump/***a***ttachment) that starts with a cascade of phosphorylations, retrieves junction-associated molecules from the plasma membrane, and ends up detaching the cell (Contreras et al., 1999, 2004). We now assay the P→A through occludin, β-catenin and β-subunit of $Na^+$,$K^+$-ATPase, and find (i) that upon treatment with ouabain, W cells retrieve these molecules from the plasma membrane; (ii) R cells do not express such phenomenon; and (iii) when W cells are in contact with R ones, they cannot express the P→A mechanism.

1882

**Expression of Connexin (Cx) 37 in Sheep Corpora Lutea during the Estrous Cycle and Prostaglandin $F_2\alpha$ (PGF)-Induced Luteal Regression**

E. Borowczyk, M. Johnson, L. P. Reynolds, D. A. Redmer, J. J. Bilski, A. T. Grazul-Bilska; Animal and Range Sci., North Dakota State Univeristy, Fargo, ND

Corpora lutea (CL) exhibit periodic growth, differentiation and regression during each estrous cycle. Gap junctions seem to play an important role in the regulation of luteal function. Ovine Cx37 was cloned and characterized (Accession # AY45977) to design species-specific probes and primers. CL collected on days 5, 10 and 15 of the estrous cycle and at 0, 4, 8, 12 and 24 h after induction of luteal regression were snap-frozen for isolation of total cellular RNA (tcRNA), and also were fixed for Cx37 immunostaining and image analysis. Luteal regression was induced by one injection of Estrumate (analog of PGF, 250 mg/injection) on day 10 of the estrous cycle. Cx37 mRNA expression was quantified and normalized to expression of the 18s ribosomal housekeeping gene by real time RT-PCR (ABI Prism 7000; Applied Biosystems, Foster City, CA). Staining of Cx37 was punctate and localized on the luteal cell borders. Cx37 mRNA and protein expression were the highest ($p<0.05$) on day 5, lower on day 10 and least on day 15 of the estrous cycle. PGF-treatment decreased ($p<0.001$) Cx37 protein but not mRNA expression at 4, 8, 12 and 24 h by 25-67% compared with 0 h (100%). However, the pattern of decrease of Cx37 mRNA and protein expression after PGF-treatment was similar. This study demonstrates that expression of Cx37 depends on the stage of luteal development, differentiation and regression and suggests a role of Cx37 in luteal function. Knowledge of the pattern of connexin expression, coupled with studies of the functional consequences of connexin expression, may lead to improved methods of estrous cycle regulation and ways to influence fertility in humans and domestic livestock. *Supported by USDA NRICGP 2002-35203-11643 grant to ATGB.*

1883

**Anti-Proliferation Activity of the Transfected Cx26 in Breast Cancer Cells**

H. J. Lee, S. K. Rhee; Biochemistry, Yeungnam University, Kyongsan, Republic of Korea

Connexin (Cx) genes are reported to be tumor suppressors in various cancer cells. Recent studies have shown that the tumor-suppressing activity acquired by Cx gene transfection is not mainly due to the recovery of the gap junction-mediated intercellular communication (GJIC). In order to

elucidate the mechanism of the Cx-induced tumor-suppressing activity, we transfected Cx26 cDNA into a rodent breast tumor cell-line (BICR-M1Rk) in which Cx43 is normally expressed and a typical pattern of GJIC had been observed. Under observation with an immuno-fluorescent microscope, the exogenous Cx26 was mainly localized in the nuclear region, whereas most of the endogenous Cx43 resided at the plasma membrane of the BICR-M1Rk cells. Consistent with the localization of the exogenous Cx26, GJIC was not increased upon transfection of Cx26 when assessed by a scrape-loading dye transfer technique. A conventional [3H]-thymidine incorporation study showed that the growth rate of the Cx26-transfected cells was significantly decreased (35%), compared to that of the control BICR-M1Rk. Taken together, our results demonstrate that the transfected Cx26 in the BICR-M1Rk cancer cell-line exerts an anti-proliferate activity in a GJIC-independent manner.

1884

### Connexin-integrated Liposomes: A Novel Tool for Drug Delivery into Mammalian Cells

M. Kaneda,[1,2] S. M. Nomura,[2,3] K. Nakahama,[1] K. Akiyoshi,[2,3] I. Morita[1,2]; [1]Department of Cellular Physiological Chemistry, Tokyo Medical and Dental University, Tokyo, Japan, [2]21st Century COE, Tokyo Medical and Dental University, Tokyo, Japan, [3]Department of Organic Materials, Institute of Biomaterials and Bioengineering, Tokyo, Japan

Intercellular communication through gap junction (GJ) plays important roles in development, proliferation, and homeostasis. GJ allows small molecules (up to 1.5kD) to pass directly from cell to cell. Here, we developed a new drug delivery system using giant liposomes with hemichannel composed of connexin43 (Cx43), a component protein of GJ. Cx43 was expressed in giant liposomes by cell-free protein synthesis. Western blot analysis showed that synthesized Cx43 was integrated into liposomal membrane, whereas enhanced green fluorescent protein, water-soluble protein, was detected in supernatants. The function of liposomes expressing Cx43 (Cx43-liposome) was examined by bidirectional dye-transfer assay. First, Cx43-transfected U2OS cells, human osteosarcoma cell line, were preincubated with calcein-AM, after that, Cx43-liposomes were plated onto the cells. 2h after, calcein was transferred from the cells to Cx43-liposomes. Second, to elucidate dye-transfer from Cx43-liposomes to cells, calcein was entrapped in Cx43-liposomes and the liposomes were applied to the Cx43-transfected cells. Calcein in the Cx43-liposomes was transferred to the cells, whereas liposomes lacking Cx43 didn't transfer. Furthermore, we examined whether a bioactive substance entrapped in Cx43-liposome can affect cellular function in Cx43-expressing cells. Treatment of oligo-peptide (TALDWSWLQTE, MW 1.3kD, IKK inhibitor) entrapped in Cx43-liposomes suppressed IL-1β-induced NF-κB activation and cyclooxygenase-2 expression, and in this system the addition of an inhibitor of GJ intercellular communication abolished this suppressive effect. The results indicate the peptide acts on the cells through GJ between cells and liposomes. In conclusion, liposome with GJ hemichannel provides a novel drug delivery carrier for transfer of water-soluble drugs to cells directly, and this is a beneficial tool for regulation of cell functions.

## Structure & Function of Membrane Proteins I (1885-1905)

1885

### A Role for Serine-373 in Regulation of the Sodium-Dependent Choline Transporter

S. A. G. Black,[1,2] R. J. Rylett[1,2]; [1]Cell Biology, Robarts Research Institute, London, ON, Canada, [2]Physiology and Pharmacology, University of Western Ontario, London, ON, Canada

Choline uptake into cholinergic neurons by the sodium-dependent choline transporter (CHT) is an important rate-limiting and regulatory step in acetylcholine synthesis. CHT cycles rapidly between the cell surface and intracellular vesicles, suggesting that trafficking of CHT is an important mechanism determining the number of transporters at the surface for uptake of choline. CHT is phosphorylated, and treatments to alter phosphorylation events change the number of functional CHT proteins at the surface, although the mechanism(s) involved remain undefined. We are examining the role of phosphorylation by protein kinase-C (PK-C) in regulation of CHT activity and subcellular localization. Using transiently-transfected HEK293 cells, we evaluated protein phosphorylation, choline uptake, and cell-surface expression of a FLAG-tagged rat CHT protein (WT-FLAG-CHT) and also that of PK-C phospho-site mutant proteins S373A-, S367A-, and T558A-FLAG-CHT. Phosphorylation, measured by [$^{32}$P]-phosphate incorporation, was enhanced for S373A-FLAG-CHT compared to WT; phosphorylation of S367A- and T558A-FLAG-CHT was not different from WT. Choline uptake of S373A-FLAG-CHT was reduced to ~50% of WT-FLAG-CHT uptake, whereas uptake of the other mutants did not differ from WT. Treatment with phorbol ester to activate PK-C reduced choline uptake of WT-, S367A-, and T558A-FLAG-CHT, but did not alter S373A-FLAG-CHT uptake. To evaluate cell-surface levels of CHT proteins, cell-surface biotinylation was used, revealing a dramatic decrease in the amount of S373A-FLAG-CHT present at the plasma membrane compared to WT. These findings indicate that mutation of serine-373 to alanine to prevent phosphorylation at this residue enhances CHT phosphorylation overall while decreasing its solute transport activity and cell-surface localization, highlighting a role for serine-373 in maintenance of normal CHT function and relative subcellular distribution, as well as its response to phorbol ester. (Supported by CIHR grant to RJR and OGSST scholarship and CIHR doctoral research award to SAB)

1886

### Characterization and Regulation of the Membrane Sector of the Vacuolar ATPase

A. R. Flannery,[1] L. A. Graham,[1] T. H. Stevens[2]; [1]Institute of Molecular Biology, University of Oregon, Eugene, OR, [2]Department of Chemistry, University of Oregon, Eugene, OR

The vacuolar ATPase (V-ATPase) is responsible for the acidification of intracellular organelles in eukaryotes. In the yeast *Saccharomyces cerevisiae*, the V-ATPase's primary function is to acidify the vacuole. It is composed of 14 subunits that are divided into two distinct domains, $V_1$ and $V_0$. The peripheral $V_1$ domain hydrolyzes ATP and couples this activity to proton transport. The $V_0$ domain contains the proton pore and is composed of six unique subunits (a, d, e, c, c', and c"). The c, c', and c" subunits are integral membrane proteins thought to form a ring ($c_{4\text{-}5}$, c', c") that rotates as ATP is hydrolyzed by $V_1$ to translocate protons across the lipid bilayer into the lumen of the vacuole. The stoichiometry of the c subunit ring has not been defined. Previous studies have shown that there is one copy each of c' and c" subunit per complex, and multiple copies of the c subunit. The number of c subunits per complex still remains to be determined. In yeast, it is thought that there are 4 to 5 copies of the c subunit per complex. To address this question, we have constructed genetic fusions of the c, c', and c" subunits. We have found that genetic fusions of c" and c subunits, c' and c subunits, as well as c" and c' subunits, are expressed as intact dimer proteins and each results in a fully assembled and functional V-ATPase complex. Our studies also reveal that genetic fusions of two c subunits also result in intact dimer proteins and a functional

complex. From these data we conclude that the V-ATPase can function with an even number of c subunits, supporting the model that there are 4 c subunits per complex.

1887

**The Cytosolic Domain of Bcl-2 Protein Forms Pores of Various Sizes in a Model Mitochondrial Outer Membrane After Acidic Ph-induced Conformation Change, Homo-association and Membrane Insertion**

J. Peng,[1] C. Tan,[1] G. Roberts,[2] S. Lapolla,[1] D. Andrews,[2] J. Lin[1]; [1]Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, [2]Biochemistry, McMaster University, Hamilton, ON, Canada

Bcl-2 family proteins regulate apoptosis by regulating the permeability of mitochondrial outer membrane. The pro-apoptotic Bcl-2 family member Bax can release Cyt c from purified mitochondria, and form large pores in the liposomal membrane that can release 2 MDa dextran molecules. Though previous studies demonstrated that the anti-apoptotic Bcl-2 family members Bcl-2 can form pores in liposomal membranes, the size of the pores and the mechanism of the pore formation remain elusive. By using spectroscopic approach, we found that the radius of Bcl-2 pores in liposomal membrane is less than 22 Å, much smaller than that of Bax pore. We also found that Bcl-2 forms pores possibly through homo-association. We demonstrated that homo-association of Bcl-2 in solution facilitates Bcl-2-membrane interaction and pore formation by showing that a preformed Bcl-2 oligomer has a faster pore-forming kinetics than a Bcl-2 monomer, that a mutant Bcl-2 having a higher affinity for homo-association than the wild type Bcl-2 also has a faster pore-forming kinetics, and that a Bax BH3 peptide which inhibits Bcl-2 homo-association also inhibits Bcl-2-membrane interaction and pore formation. In contrast to a previous report, we found that the low pH that is required for Bcl-2 to form pores in the liposome system did not promote Bcl-2 homo-association. Instead the low pH induced a conformation change in Bcl-2 that exposes the hydrophobic core of Bcl-2 to solvent, promoting Bcl-2-membrane interaction. Interestingly the Bcl-2 mutant with fast pore-forming kinetics in vitro shows a better activity in Rat-1 cells against etoposide-induced apoptosis. Based on these data, we proposed a model mechanism for Bcl-2 pore-formation that may be used by Bcl-2 to regulate the permeability of membrane during apoptosis.

1888

**High-Level Heterologous Expression and Purification of Functional Human Vitamin K 2,3-Epoxide Reductase (hVKORC1): The N-terminus is Inaccessible to in situ Labeling in Membranes**

C. Reinhart,[1] W. Haase,[2] S. Rost,[3] A. Fregin,[3] M. Huenerberg,[3] C. R. Mueller,[3] C. G. Bevans,[2] J. Oldenburg[3,4,5]; [1]Molecular Membrane Biology Department, Max Planck Institute of Biophysics, Frankfurt/Main, Germany, [2]Structural Biology Department, Max Planck Institute of Biophysics, Frankfurt/Main, Germany, [3]Institute of Human Genetics, Biocenter - University of Wuerzburg, Wuerzburg, Germany, [4]Institute of Transfusion Medicine and Immunohematology, DRK Blood Donor Service Baden-Wuerttemberg-Hessen, Frankfurt/Main, Germany, [5]Institute of Experimental Hematology and Transfusion Medicine, University Clinic Bonn, Bonn, Germany

The trace nutrient vitamin K is rapidly converted to its 2,3-epoxide (VK>O) in animal cells during the initial enzymatic step leading to post-translational ?-carboxylation of various intracellular proteins. Recycling of VK>O to vitamin K is achieved by a putative vitamin K epoxide reductase (VKOR) enzyme complex (*Arch. Biochem. Biophys.* 141:473). The recently identified human *VKORC1* gene (*Nature* 427:53*7, Nature* 427:541) encodes hVKORC1 (18.2 KDa, 163 amino acids), an ER intrinsic membrane protein. In order to obtain sufficient quantities of functional hVKORC1 for structural studies, we produced up to 3.47 mg/L affinity-tagged hVKORC1 in *Pichia pastoris*. VKOR activity of membranes from cells with multi-copy constructs pPIC9K(αF-flag-his$_{10}$-VKORC1-biotag) was measured using a standard assay (*J Clin Invest* 76(5):1879) and was several hundred-fold greater than that of cells with an empty vector. In order to enhance target purity in membranes prior to solubilization, we determined the subcellular localization of hVKORC1. Attempts to visualize hVKORC1 subcellular localization by electron microscopic examination of immunostained thin section and freeze-fracture specimens of construct-expressing cells revealed no significant immunostaining, suggesting no construct expression or construct proteolysis. However, Western blots of SDS-solubilized membranes from the same cells indicated high levels of construct. To further investigate these paradoxical results, we performed preparative sedimentation of cell extracts, under isotonic conditions, to obtain ER/plasma membranes and membrane-bound organelles. Surprisingly, only 20% of hVKORC1 construct was found by Western blot in the ER/plasma membrane fraction, while most of the remaining construct was found in the mitochondrium/peroxisome/vacuole fraction. Taken together, these results suggest that the aqueous-soluble N-terminal domain of hVKORC1 is folded *in vivo* into an adjacent protein domain or might participate in binding interaction with other proteins. Support: NationalGenomeResearchNet CardiovascularDiseases(BMBF-DLR-01GS 0424)&BaxterGermany

1889

**Topological Analysis of the Human ABC Transporter ABCG2**

N. K. Diop; Chemistry, Purdue University, West Lafayette, IN

The human ATP Binding Cassette (ABC) transporter ABCG2 is a 655 amino acid glycoprotein that can confer multidrug resistance to cancer cells by functioning as an energy dependent efflux pump. We have previously shown that ABCG2 is N-linked glycosylated at asparagine (Asn) 596 and that the lack of glycosylation does not affect function or cell surface localization. These data provided the first direct evidence for the localization of the loop containing Asn 596 to the extracellular space, previously only predicted from hydropathy analysis. In our present work, we used an epitope insertion approach to further test the topology model of ABCG2. Using the glycosylation deficient mutant ABCG2 (N596Q) as a template, we introduced fourteen N-linked glycosylation consensus sites into the predicted intracellular and extracellular loops of ABCG2. Flow cytometric analyses show that the majority of our mutants are expressed at the cell surface and are capable of transporting rhodamine 123. Among the engineered glycosylation mutants, three (G577N, F586S, and A606T) are glycosylated and treatment with peptide N-glycosidase F reduces the apparent molecular mass by SDS-PAGE. These data indicate that these residues are localized in the extracellular space. In addition, we introduced FLAG epitope tags at either the amino or carboxyl terminus of ABCG2 and found that these sequences were localized to the cytoplasm. Together, these data support a topological model of ABCG2 containing six transmembrane segments, three extracellular loops, and both the amino and carboxyl termini localized in the cytoplasm.

1890

**Multi-site Occupancy of the Cytochrome P450 Catalytic Cavity by Substrates and Inhibitors**

A. Banerjee, H. Liu; Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI

The cytochrome P450 monooxygenases form a large and omnipresent superfamily of enzymes. While each individual P450 exhibits unique and specific substrate and catalytic activity profiles, overall, P450s perform a wide range of oxidation reactions on a diverse spectrum of substrates. The mammalian microsomal P450s degrade most xenobiotics encountered by man and animals. P450s are important targets for drug design. In our laboratory, structure-based ligand design has been used to find putative inhibitors of selected CYPs. Virtual screening of large libraries can be performed speedily *in silico*, and the best results can then be tested for biological activity in experimental assay systems. In the current work, the paradigm of structure-based ligand design was followed in which the aim was to design chemical inhibitors. We have screened ~450,000 molecules of ACD 3D database (MDL, Inc.) using LUDI software (Accelrys, Inc.). The radius and center of search depended on the CYP target pockets. We fixed a radius of 12Å from the heme iron for different targets. By our studies we have found the existence of a second compound binding site at the proximal side of heme; this we refer to as the "2nd site" where as the catalytic site is the "1st site". We have also performed enzyme inhibition assays with the compounds. We have used Gentest CYP-2E1, -2C9 and -3A4 high throughput inhibition assay kits to measure the potential inhibition or induction of the CYP2E1, -2C9 and -3A4 catalytic activity. The results will be presented. For CYP2C9 we find that selected compounds can actually increase its catalytic activity. Crystallographic determination of their binding properties and changes they induce in these CYPs will be very illuminating for the study of CYP structure and function relationships. (Supported by NIH grant GM-59467.)

1891

**Identification of the Domains of Heavy Chain Subunits of Heterodimeric Amino Acid Transporters in the Recognition of their Light Chains**

N. Ahmed, P. Kongsuphol, Y. Kanai; Dept. of Pharmacology and Toxicology, Kyorin University, Mitaka, Japan

The heterodimeric amino acid transporters are comprised of a 12-membrane-spanning light chain subunit of SLC7 family and a single-membrane-spanning type II membrane glycoprotein of SLC3 family as a heavy chain subunit. The heterodimerization of two subunits are proposed to be required for the functional expression of the transporters in the plasma membrane. 4F2hc (4F2 heavy chain) subserves various amino acid transport systems by dimerization with six different light chains, whereas one light chain is associated with other heavy chain subunit rBAT (related to $b^{0,+}$ amino acid transporter). In order to determine the mechanisms of recognition of the light chain subunits by the heavy chains, we have generated chimeras of human 4F2hc and human rBAT and examined whether they are associated with LAT1 a partner of 4F2hc or $b^{0,+}$AT/BAT1 a partner of rBAT. The chimeras were functionally expressed in *Xenopus oocytes* or COS-7 cells and analyzed by measuring the ability to induce amino acid transport activity. The results indicated that the transmembrane and cytoplasmic juxta-transmembrane region of 4F2hc and rBAT are essential for the recognition of LAT1 and $b^{0,+}$AT/BAT1, respectively. The importance of this region was further confirmed by the series of complementary chimeras (rBAT/4F2hc chimeras). It is, thus, suggested that the recognition of light chains by heavy chains is accomplished by the interaction of amino acid residues of heavy chains in transmembrane and intracellular small region close to the transmembrane domains with those of the transmembrane and intracellular domains of light chains.

1892

**Novel Glucose Transport Activation Process Regulated by Glucose in C2C12 Myotubes**

T. Nedachi, M. Kanzaki; Tohoku University Biomedical Engineering Research Organization, Miyagi, Japan

It is well established that insulin stimulates glucose uptake in skeletal muscle by causing translocation of the insulin responsive glucose transporter GLUT4 from intracellular storage sites to the plasma membrane. However, the established skeletal muscle cell lines, such as L6 and C2C12, usually show very low insulin-dependent glucose uptake/GLUT4 translocation compared to skeletal muscle *in vivo*. In this study we attempted to characterize glucose transport system in C2C12 myotubes. **[Methods]** Glucose transport activity was measured by 2-deoxyglucose (2DG) uptake, and GLUT4 translocation was assessed by the exofacial-myc-GLUT4-ECFP translocation evaluated by anti-Myc Ab uptake assay. **[Results]** Even in the basal condition, C2C12 myotubes displayed considerably high 2DG uptake without any detectable GLUT4 protein at the plasma membrane. After insulin stimulation, about 3-fold increase in GLUT4 translocation was induced, whereas only a little (~1.2-fold) of insulin-stimulated 2DG uptake was observed, indicating that there is obvious discrepancy between GLUT4 translocation and glucose uptake. Interestingly, the basal 2DG uptake, which is not mediated through GLUT4, was rapidly decreased by the 5-min-pretreatment of the myotubes with 22.5 mM glucose (pre-HG), while it was increased by glucose deprivation. The attenuation of 2DG transport activity by the pre-HG was not due to a competitive inhibition by glucose, but required enzymatic activites, since the inhibitory effect of pre-HG was not observed at 4C. Importantly, a PI 3-kinase inhibitor wortmannin also suppressed the basal 2DG uptake to a similar extent to the pre-HG. **[Conclusion]** Based upon these data, we here propose that in C2C12 myotubes there is unidentified regulatory mechanism for glucose transport activity, which is dependent on glucose metabolism and is also sensitive to wortmannin. This glucose transport activation process modulated by glucose perhaps obscures the insulin-induced glucose uptake via GLUT4 translocation in cultured myotubes.

1893

**Structural Changes of Band 3 in Miltenberger Blood Group-expressed Erythrocytes**

K. Hsu, Y. Chen, Z. Chen, M. Lin; Transfusion Medicine Laboratory, Mackay Memorial Hospital, Tamsui, Taiwan Republic of China

Various Taiwanese aboriginal tribes exhibit significantly higher frequencies of the rare Miltenberger (MNS) blood group expressions. The MNS molecular entities, originated from specific gene recombination of glycophorin A and glycophorin B, are distinct glycophorin hybrids that could elicit hemolytic reactions during incompatible blood transfusion. As there are a plethora of Miltenberger phenotypes (11 variations serologically identified up to date), we attempt to purify these unique glycophorin complexes and dissect their compositions using proteomic approaches. Glycophorin-band 3 macrocomplexes were antibody-purified from red blood cell ghost. Their individual components were identified by 1D SDS-PAGE and Western blots. Further separation of the glycophorin macrocomplexes using 2D gel electrophoresis shows no apparent difference between Miltenberger type III (Mi.III) and normal samples when visualized by conventional silver stain. Nonetheless, Miltenberger band 3, once 2D gel electrophoresed, is no longer recognizable on Western blot, whereas normal band 3 still is. As this phenomenon is not apparent in SDS-

solubilized protein extracts, we hypothesize that the 3D structure of Mi.III band 3 probably differs from that of normal band 3 due to its physical interaction with the glycophorin hybrid of Mi.III. We are currently actively pursuing the structural and functional consequences resulted from these glycophorin hybrids characteristic of Miltenberger.

1894

**Characterization of a Thyroid Sodium/Iodide Symporter (NIS) Mutant (V59E) That Causes Congenital Iodide Transport Defect (ITD)**

M. D. Reed, C. S. Ginter, N. Carrasco; Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY

Iodide (I-) is an essential component of the thyroid hormones T3 and T4, which are required for proper development of the central nervous system and for intermediary metabolism in virtually all tissues. Most of the ingested I- is actively transported into thyroid epithelial cells via NIS, a specialized intrinsic plasma membrane glycoprotein that couples the "downhill" inward translocation of sodium to the "uphill" inward translocation of I-. The cDNA that encodes NIS was first identified in our laboratory, where an extensive structure/function characterization of NIS is being carried out. Several NIS mutants have been identified as causes of congenital ITD, including a mutant that contains glutamic acid instead of valine at position 59 (V59E). ITD is characterized by low thyroid I- uptake, hypothyroidism, goiter, and a low saliva/plasma I- ratio. The patient's V59E substitution yields a non-functional NIS protein; the aim of this project was to characterize its effects and structural/functional implications. I have transfected COS-7 cells with various NIS constructs coding for different amino acids (A, D, E, I, K, L, M, N, Q, R, T) at position 59, and have characterized the effects of each substitution on protein expression, processing, targeting to the plasma membrane, and I- transport activity. Transfection of all mutant NIS constructs resulted in proper expression, processing, and targeting of NIS. Kinetic analysis of A, I, L, M, N, Q, and T showed a decrease in iodide uptake due to a Vmax effect, not a change in the affinity of NIS for Na or I-. Constructs encoding a charged amino acid or proline at position 59 showed no I- transport activity. In conclusion, the structural requirement for NIS at this position is a neutral, helix-promoting, branched amino acid.

1895

**Molecular Dissection of the Syntaxin4/Munc18c Interaction: Implications for the Regulation of Membrane Trafficking**

J. A. Lopez,[1] C. F. Latham,[2] D. H. Blair,[1] C. L. Gee,[2] S. Hu,[2] E. Westbury,[2] N. J. Bryant,[1] J. L. Martin,[2] D. E. James[1]; [1]Diabetes and Obesity, Garvan Institute of Medical Research, Sydney, Australia, [2]Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia

SNARE proteins play a fundamental role in docking and fusion of membranes at every compartment throughout the cell. Regulation of SNARE complex assembly is a critical control point in intracellular membrane trafficking. A key player in this regulation is the Sec1p/Munc18 (SM) family of proteins. There is considerable debate, however, concerning the precise role of different SM isoforms in the SNARE assembly process. One model based on secretory neuronal SNAREs, suggests that the SM protein stabilises the entire cytoplasmic domain of syntaxin in a closed form, preventing SNARE assembly and fusion. An apparently contradictory model, based on intracellular SNAREs, suggests that the SM protein can bind both monomeric syntaxin and SNARE ternary complexes. The aim of this study was to define the interaction of the plasma membrane t-SNARE, Syntaxin4, and its cognate SM protein, Munc18c. Consistent with the latter model we found deletion of Syntaxin4's first 29 amino acids resulted in almost complete loss of Munc18c binding. In addition, mutation of the highly conserved leucine 8 in Syntaxin4 resulted in a 70% reduction in Munc18c binding. We also found that Munc18c interacts with preformed SNARE complexes *in vitro*. Interestingly, however, Munc18c/Syntaxin4 dimeric complex formation prevented subsequent binding of Syntaxin4 with its SNARE partners SNAP23 and VAMP2. Taken together these results suggest that the Munc18c/Syntaxin4 interaction occurs via sequential binding initiated through Syntaxin4's $NH_2$-terminus. This may chaperone Syntaxin4 through the *cis* to *trans* SNARE complex transition. Supporting a chaperone-type role, we found RNAi knockdown of Munc18c resulted in a coincident reduction in Syntaxin4 levels. These data are consistent with a model whereby Munc18c regulates the assembly of the functional SNARE complex through both negative regulation of Syntaxin4 prior to fusion and by ensuring the fidelity of SNARE assembly/disassembly.

1896

**The Putative Membrane Occupation Repeat Nexus (MORN) Region of Arabidopsis PtdInsP Kinase 1 Affects Enzyme Activity as Well as Subcellular Distribution**

Y. Im, A. J. Davis, E. Johannes, N. S. Allen, W. F. Boss; Plant Biology, North Carolina State Univ, Raleigh, NC

The type I B family of phosphatidylinositol phosphate kinases (PIPKs) from Arabidopsis is characterized by a region of N-terminal putative MORN repeats. In theory, this MORN region should bind to membranes and affect the subcellular distribution of the enzyme. We have used in vitro and in vivo studies to characterize the function of the putative MORN region of *At*PIPK1(At1g21980). Constructs encoding both truncated and full length proteins were expressed in *E. coli* and the recombinant proteins were characterized for enzyme activity. Deletion of the N-terminal putative MORN region (amino acid #1-252) increased the activity of the remaining truncated C-terminal catalytic domain (#253-752) >4 fold. When the recombinant MORN region was added back to the truncated C-terminal catalytic domain, it enhanced the Vmax of the truncated C-terminal enzyme but it had no effect on activity of the full-length enzyme suggesting that the MORN region facilitated substrate delivery only when the active site was available. The MORN region affects the expression and localization of *At*PIPK1 and expressing the GFP-MORN region increases the PtdInsP kinase activity of the plasma membrane. Our working hypothesis is that the putative MORN region of *At*PIPK1 regulates enzyme activity as well as subcellular distribution. Lipid binding studies indicate that the MORN region binds to both the substrate, PtdIns(4)P, and the product, PtdIns(4,5)$P_2$, of the lipid kinase reaction. Our current model is that the MORN region protects the active site of the lipid kinase until the enzyme (particularly the MORN region) associates with a lipid bilayer; then the catalytic domain opens up and the MORN delivers PtdIns(4)P and removes PtdIns(4,5)$P_2$. This work was supported in part by a grant from the National Science Foundation.

1897

**Role of the C-Terminus in the Membrane Trafficking of hSERT**

B. Jeffus, F. Kilic; Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR

The human serotonin transporter (hSERT) is the primary means of attenuating serotonergic signaling in the central nervous system of humans. hSERT exists as an oligomeric protein that spans the plasma membrane 12 times and where both termini are cytosolic. Included in the same protein

family as hSERT, are other $Na^+$ and $Cl^-$ dependent monoamine transporters, namely the human dopamine transporter (hDAT) and the human norepinephrine transporter (hNET). In addition to belonging to the same family, hSERT, hNET, and hDAT share 60% sequence homology and have many overlapping characteristics. Previous research has indicated the importance of the C-terminus of hNET and hDAT in the regulation of their transport function. Considering this, and the shared sequence homology to hSERT, we have investigated the role of the C-terminus in the function of hSERT proteins. Through the use of genetic, immunofluorescent, and functional assays we have identified amino acid residues in the C-terminus that are vitally important to the transport function of hSERT proteins. In addition to this, we believe the alteration in transport activity is due to an inability of cellular machinery to properly traffic the proteins to the plasma membrane, not due to an altered functional state of the protein.

1898

**Functional Characterization of a Palmitoylation-Deficient CD81 Mutant**

C. Delandre, T. R. Penabaz, A. L. Passarelli, R. J. Clem, S. K. Chapes; Division of Biology, Kansas State University, Manhattan, KS

Functional characterization of a palmitoylation-deficient CD81 mutant The transmembrane protein CD81 is a member of the tetraspanin superfamily that regulates the assembly of the B-cell coreceptor (CD19/21/81) and serves as a receptor/ coreceptor for hepatitis C virus and *Plasmodium falciparum*. CD81 may function as an adaptor molecule which regulates how proteins such as integrins, antigen receptors, and other tetraspanins associate in the plasma membrane microdomains. Recently, several groups showed that tetraspanin palmitoylation linkage of long-chain fatty acids to cysteine residues may have an important role in the formation of these microdomains. In addition, we hypothesized that palmitoylation is involved in the participation of CD81 in microdomain assembly. To test this hypothesis, we generated a palmitoylation-deficient CD81 mutant (CD81plm) by altering all available cysteines in the cytoplasmic and transmembrane domains. We found that CD81plm could be expressed in mammalian cells while maintaining antigenicity as determined by a conformation specific CD81 antibody. The migration of CD81plm in SDS-PAGE was consistent with the lack of palmitic acid modification. We tested the palmitoylation state of this mutant using radiolabelled palmitic acid, and as expected, no detectable palmitic acid was incorporated in CD81plm. CD81plm was transported to the plasma membrane as indicated by flow cytometry data. Finally, we found that there is an association between CD81plm and the tetraspanin CD9, a major CD81 binding partner. In conclusion, we have generated an important tool that will bring new insights into the regulation of CD81 by palmitoylation. This project was funded by NIH grants AI0552206, AI55052, RR16475, and NASA grants NAG2-1274 and NAGW-1197 and the Kansas Agriculture Experiment Station.

1899

**Localization of PAT Family Proteins in Cholesterol-Loaded Macrophages and Adipocytes**

O. Hofnagel,[1] I. Buers,[1] M. J. Robenek,[2] D. Troyer,[1] N. J. Severs,[3] H. Robenek[1]; [1]Department of Cell Biology and Ultrastructure Research, Leibniz-Institute for Arteriosclerosis Research, University of Münster, Germany, [2]Department of Medicine, Division of Cardiology, Pulmonary Disases and Angiology, University of Düsseldorf, Germany, [3]National Heart and Lung Institute, Imperial College of London, United Kingdom

The PAT family proteins, named after perilipin, adopophilin and TIP47, are implicated in intracellular lipid metabolism. Current information on the localization of PAT family proteins is inconsistent. The general consensus is that these proteins occur exclusively in the surface monolayer of lipid droplets and not in any other subcellular compartment. We studied the distribution of the PAT family proteins in cholesterol-loaded THP-1 cell derived macrophages and 3T3-L1 adipocytes using freeze-fracture immunolabeling and other techniques. We now demonstrate that in macrophages and adipocytes PAT family proteins are: 1) distributed not only in the surface but also throughout the lipid droplet core; 2) localized in endoplasmic reticulum (ER) and outer nuclear membranes, and 3) integral components of the plasma membrane. The localization of PAT family proteins apart from lipid droplets to the plasma membrane and membranes of organelles such as ER and nucleus raises intriguing questions as to its roles at these sites. Under normal culture conditions, PAT family proteins are dispersed in the cytoplasmic leaflet of the plasma membrane. Stimulation of lipid droplet formation by cholesterol-loading of the cells leads to clustering of the PAT family proteins in specialized plasma membrane domains. The aggregation of the PAT family proteins into such assemblies may facilitate carrier-mediated lipid influx from the extracellular environment into the lipid droplet. They further raise the possibility that transit of PAT family proteins with their fatty acid cargo into lipid droplets functions as part of this influx mechanism. Whether the pools of PAT family proteins in ER and nuclear membranes are regulated by alternative mechanisms to those of the plasma membranes and whether they provide a pathway by which PAT family proteins transit to the lipid droplet remains to be determined. Supported by the Deutsche Forschungsgemeinschaft, SFB 492.

1900

**Role of the Membrane Lipid Structure in Signaling Through G Protein-coupled Receptors**

J. Casas,[1] J. Martínez,[1] O. Vögler,[1] Q. Yang,[2] R. Alemany,[1] G. Borchert,[1] T. Nagy,[1] S. M. Lanier,[3] P. V. Escribá[1]; [1]Biology, University of the Balearic Islands, Palma de Mallorca, Spain, [2]Medicine, Medical University of South Carolina, Charleston, SC, [3]Health Science Center, Lousiana State University, New Orleans, LA

Heterotrimeric G proteins are peripheral membrane proteins that propagate signals from membrane receptors to regulatory proteins localized in distinct cellular compartments. The aim of our study was to determine the role of the membrane lipid structure in the interaction and activity of G proteins and related proteins. For this purpose, we used two systems: model membranes (liposomes) and membranes from 3T3 cells overexpressing the $\alpha_{2A/D}$-adrenoceptor. We found that nonlamellar lipid phases favor the binding of pre-active Gαβγ proteins, driven by the Gβγ dimer. Activated Gα subunits dissociate from Gβγ dimers. Gα subunits have higher affinity for lamellar-prone regions, such as lipid rafts, where they interact with signaling effectors (e.g., adenylyl cyclase). Gβγ dimers remain in $H_{II}$-prone regions nearby the receptor and recruit GRK, which inactivates the receptor protein. Thus, membrane structure is involved in recruitment of G proteins to the receptor vicinity and also participates in the active sorting of G protein subunits upon activation by agonists. In addition, the membrane lipid structure also regulates the function of G proteins and related signaling proteins. In this context, the oleic acid (OA) changed the membrane structure and regulated G protein activity in 3T3 cell membranes. OA also induced marked changes in $\alpha_{2A/D}$-adrenoceptor and adenylyl cyclase activities. In contrast, the OA analogs elaidic and stearic acids did not change the activity of the above-mentioned proteins. In contrast, elaidic and stearic acids did not significantly change membrane lipid organization or function of G proteins, receptors and effectors. These fatty acids are chemical but not structural OA analogs, supporting the

structural basis of the modulation of membrane lipid organization and subsequent regulation of G protein-coupled receptor signaling. The present results demonstrate the active participation of membrane lipid structure in cellular signaling.

1901

### Lipid Therapy: Manipulation of the Membrane Structure for Treatment of Human Pathologies

P. V. Escribá,[1] J. Martínez,[1] A. Gutierrez,[2] V. Lladó,[1] F. Barceló,[1] J. Prades,[1] J. Casas,[1] J. Besalduch,[2] R. Alemany,[1] O. Vögler[1]; [1]Biology, University of the Balearic Islands, Palma de Mallorca, Spain, [2]Hematology, Hospital Son Dureta, Palma de Mallorca, Spain

Most marketed drugs are targeted to proteins. Some novel approaches aim other cellular structures. We have developed a new therapeutic approach based on the modulation of the membrane lipid structure: **Lipid Therapy**. The goal of this work was to study the molecular bases underlying the control of cell functions upon manipulation of membrane structure and subsequent clinical applications. We have designed a fatty acid, 2-hydroxy-9-cis-octadecenoic acid (Minerval), and studied its effects on the structure of lipid membranes by X-ray scattering. We have also investigated its activity on cancer cell proliferation in cultured cells and animal models of cancer. Finally, we have investigated protein expression alterations produced by Minerval treatments on cancer cells by immunoblotting and RT-PCR. We found that Minerval increased the nonlamellar-phase propensity of membranes and subsequent recruitment of protein kinase C (PKC) to the plasma membrane. The moderate activation of this enzyme was associated with overexpression of $p21^{CIP1}$, which in turn inactivated cyclin-cdk complexes. As a consequence, the retinoblastoma protein (pRb) was hypophosphorylated, favoring formation of pRb/E2F-1 complexes. Impairment of active E2F-1 release resulted in lack of activation of several genes involved in cell proliferation: E2F-1, cyclins, cdks and dihydrofolate reductase (DHFR). Indeed, DHFR appeared to be a pivotal protein in the effects mediated by Minerval, since addition of the enzyme product (THF) to the incubation medium reversed the antiproliferative effects of the drug. In addition to its high antitumor activity, Minerval showed a lack of toxicity and side-effects. In summary, we have used the manipulation of the plasma membrane structure to trigger a series of molecular events that resulted in antitumor activity through a novel pharmacological mechanism. This therapeutic strategy can be used for treatment of other pathologies, such as hypertension and obesity.

1902

### Molecular Basis of Oxidative Stress Protection by *Bacillus anthracis Dlp-1* and *Dlp-2*

X. Liu,[1] E. C. Theil[2,3]; [1]Center for BioIron, Children's Hospital and Research Center at Oakland, Oakland, CA, [2]Dept. of Nutritional Biochemistry and Toxicoly, University of California, Berkeley, Oakland, CA, [3]BioIron, Children's Hospital and Research Center at Oakland, Oakland, CA

Cells use ferritin for protection during stress by converting iron and oxidants to solid mineral. Bacterial pathogens resist host macrophage release of oxidants during infection. The conserved cage-like *Dps* proteins are bacterial mini-ferritins. Many bacteria overexpress *Dps* under stress conditions. Understanding cellular roles of *Dps* proteins in bacterial stress response, physiology, and pathogenesis requires study of molecular mechanisms. Objective: Biochemical characterization of *Bacillus anthracis Dlp-1* and *Dlp-2*, two isoforms of *Dps*. Results *Iron uptake/mineralization*: *Dlp-1* and *Dlp-2* catalyze $Fe^{2+}$ oxidation with $O_2$ and store up to 500 $Fe^{3+}$. Rates of $Fe^{2+}$ /$O_2$ reaction are comparable for *Dlp-1* and *Dlp-2* and are 100-fold slower than maxi-ferritins. *Dlp-2* but not *Dlp-1*, also catalyzes $Fe^{2+}/H_2O_2$ reaction 3-fold faster than with $Fe^{2+}/O_2$ reaction, illustrating its role in $H_2O_2$ detoxification. *Iron Release*: Both *Dlp-1* and *Dlp-2* control Fe efflux from dissolved mineral with reductant/chelator. Thermostability study with *CD* detected flexible subdomains, possibly iron pores, transition around 37°C. Rates of iron release correlate well with iron pore dynamics, suggesting pore gating to regulate Fe efflux. *DNA binding and protection*: *Dlp-1* bond DNA but did not protect it from hydroxyl radical damage; *Dlp-2* did not bind DNA but protected it against oxidative cleavage; *Dlp-1* and *Dlp-2* together altered the properties of DNA/protein complexes. Results reflected different roles of *Dlp-1* and *Dlp-2* interacting with DNA, and presence of both *Dlp-1* and *Dlp-2* offers comprehensive protection to bacterial genome from damage. Conclusions: The multiple biochemical functions associated with *Dlp-1* and *Dlp-2* suggest complementary roles of the two proteins in iron and oxygen homeostasis, peroxide detoxification, and DNA protection that contribute to the ability of *B. anthracis* to survive iron depletion and the "oxidative burst" of macrophages and neutrophils. Support: NIH-DK20251, Cooley's Anemia Foundation, CHORI Foundation.

1903

### Functional Relevance of Overexpression of LAT1 and 4F2hc in SHR Immortalized Renal Proximal Tubular Epithelial Cells

M. Pinho,[1] P. A. Jose,[2] P. Soares-da-Silva[1]; [1]Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal, [2]Department of Pediatrics, Georgetown University Medical Center, Washington, DC

The present work examined the expression of LAT1 and its associated glycoprotein 4F2hc in renal cortical membranes and immortalized renal proximal tubular cells from spontaneously hypertensive (SHR) and normotensive (WKY) rats. The study also examined the inward and outward transport of [$^{14}$C] L-leucine, the preferred substrate of LAT1. The [$^{14}$C] L-leucine uptake in WKY cells was largely promoted through a $Na^+$-insensitive transporter, though in SHR cells a significant component (~50%) of [$^{14}$C] L-leucine influx was found to require extracellular $Na^+$. [$^{14}$C] L-leucine uptake that was insensitive to system A inhibitor N-(methylamino)-isobutyric acid (MeAIB), but sensitive to inhibition by 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BCH), a system L inhibitor. L- isomers of neutral amino acids and D-leucine markedly reduced the [$^{14}$C] L-leucine accumulation. In SHR cells [$^{14}$C] L-leucine uptake in the presence of extracellular $Na^+$ was sensitive to inhibition by basic amino acids. Accumulation [$^{14}$C] L-leucine in SHR and WKY cells was insensitive to pH variations. Unlabeled L-leucine increased [$^{14}$C] L-leucine efflux in a time- and concentration-dependent manner. The abundance of LAT1 and 4F2hc were also found to be greater in SHR than in WKY, both in renal cortex and immortalized renal proximal tubular cells. In conclusion, it is likely that system $B^{0,+}$ might be responsible for the $Na^+$-dependent uptake of L-leucine in SHR cells, whereas the $Na^+$-independent uptake of L-Leucine in both cells lines may include system LAT1, which functions as an exchanger and whose activation results in *trans*-stimulation of L-leucine outward transfer.

1904

### The Stem-Cell Marker Bcrp1/Abcg2: a Role in Mature Sperm?

C. Scharenberg,[1] P. Repges,[1] T. Jähn,[1] T. Saharge,[1] R. W. Robey,[2] G. Wennemuth[1]; [1]Department of Anatomy and Cell Biology, Philipps-University of Marburg, Marburg, Germany, [2]Center of Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD

An increasingly exploited strategy for the isolation of stem cells from a variety of species and tissues is based on the increased efflux-capacity for

the lipophilic dye Hoechst 33342. This efflux activity is mostly mediated by Abcg2, an ATP-binding-cassette transporter, which is highly expressed in various stem cells. However, ablation of Abcg2 does not lead to a stem cell defect. In addition to its expression on stem cells, Abcg2 is also expressed by some mature cells in blood, liver, kidney, gut, and brain. While studying the expression pattern using RT-PCR from FACS-isolated germ cell populations of different developmental stages, we noted that Abcg2 is upregulated at later stages of spermatogenesis, particularly spermatocytes I and II. Western blot analysis using a polyclonal Abcg2-antibody corroborated expression of a 72 kDa band in mature sperm of murine, bull and human origin. Immunocytochemistry studies with the same antibody revealed an acrosomal staining pattern of Abcg2 in spermatozoa. Experiments using known Abcg2 substrates and the Abcg2-specific inhibitor Fumitremorgin C demonstrated efflux activity of Abcg2 in mature sperm, suggesting its function in the outer plasma membrane. Among the substrates of Abcg2 are genotoxic agents, steroids and other lipophilic molecules, some of which play important roles in capacitation of spermatozoa. Besides its putative function in prevention of genotoxic stress, we investigated whether Abcg2 mediates changes in membrane composition that go along with maturation of spermatozoa. Based on the evolutionary conservation of this ABC-Transporter in mature sperm, we conclude that Abcg2 is an important regulator of post-testicular maturation of spermatozoa.

1905

### Effects of Growth Support on Multi-Drug Resistance and Cellular Differentiation

S. D. Sheridan, A. A. Arena; Millipore Corporation, Danvers, MA

Effects of Growth Support on Multi-Drug Resistance and Cellular Differentiation Steven D. Sheridan, Ph.D. and Andrew A. Arena Multiple drug resistance due to efflux is a major cause for failure of many therapeutic regiments. For example, augmentation of transporter expression can protect cancer cells from chemotherapeutic agents. Over-expression of ATP-binding cassette (ABC) drug transporters, including MDR-1 (P-glycoprotein) and the MRP proteins, have been shown to be involved in drug resistance by preventing the uptake of oral drugs, causing the efflux of drugs from target tissues, or both. This study investigates the impact of tissue culture conditions on optimal ABC transporter expression, activity and multi-drug resistance. This study utilizes cell lines with various levels of efflux activity including Caco-2, MDCK and MDCK cells transfected with the human MDR-1 gene. Cell growth was compared on microporous filters against solid plastic plate growth supports. Monolayer integrity in the filter plates was evaluated by Trans-epithelial Electrical Resistance (TEER) and Lucifer yellow rejection. MDR-1 protein activity and expression was characterized by calcein-AM uptake, immunocytochemistry, and drug resistance measured by $LD_{50}$ determination. Transfected MDCK cells showed high MDR-1 activity that was augmented after culture on filters when compared to solid bottom plates. Both non-transfected MDCK cells and non-fully differentiated Caco-2 cells (10 days of culture or less) had minimal activity when grown on both filter and solid bottom plates. Alterations of MDR-1 activity and expression in the Caco-2 cells were dependent on growth support upon differentiation. This data suggests that microporous filter supports contribute to greater monolayer differentiation and therefore higher expression of MDR1. Filter based cell culture plates are ideal platforms for culturing differentiated cell monolayers and also provide an optimal environment for investigating the drug resistance activity of ABC transporters.

## Golgi to Cell Surface Transport (1906-1924)

1906

### The Role of Ypt/Rab GTPase Interactions in Coupling Vesicular Transport Steps

N. Segev, Z. Lipatova; Biological Sciences, University of Illinois, Chicago, IL

Ypt/Rab GTPases are master regulators of protein trafficking in eukaryotic cells. When in the GTP-bound state, they interact with multiple effectors that mediate all the known aspects of vesicular transport, from vesicle formation and motility, to their targeting and fusion. An attractive idea is that Ypt/Rabs and their accessory factors not only regulate individual vesicular transport steps, but also coordinate them. In yeast, the Ypt31/32 GTPase pair regulates the formation of trans-Golgi vesicles, while Sec4 GTPase controls their subsequent targeting and fusion. Here, we show that these GTPases interact directly in a nucleotide-specific manner, and that this interaction is physiologically relevant. Nucleotide-specific interaction of Ypt31 and Sec4 was shown by two-hybrid analysis and co-precipitation of purified proteins. The physiological relevance was shown by co-localization of these GTPases, co-precipitation from yeast lysates, and genetic interactions of nucleotide-restricted Ypt31 and Sec4 mutations. Finally, we show that, like Sec4, Ypt31/32 localize to trans-Golgi vesicles, and that Ypt31 is required for intracellular localization of Sec4. These results suggest that direct interaction of Ypt31/32 with Sec4 on trans-Golgi vesicles is important for the recruitment of Sec4 to these vesicles, which in turn is required for their polarized targeting and fusion. Therefore, direct GTPase interactions serves to couple Ypt31/32-mediated trans-Golgi vesicle formation with Sec4-mediated targeting and fusion of these vesicles. We propose direct GTPase-GTPase interactions as a novel mechanism by which the highly conserved Ypt/Rab GTPases coordinate separate vesicular transport steps.

1907

### Roles of ARFRP1 (ADP-Ribosylation Factor-Related Protein 1) in Post-Golgi Membrane Trafficking

H. Shin,[1] H. Kobayashi,[2] M. Kitamura,[1] K. Nakayama[1]; [1]Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan, [2]Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan

ADP-ribosylation factors (ARFs) constitute a family of small GTPases belonging to the Ras superfamily and play essential roles in intracellular membrane trafficking. In addition to ARFs, there exists a subfamily of small GTPases with sequence similarity to ARFs, referred to as the Arl (ARF-like) subfamily. Little is known about the function of the Arl proteins, yet Arl1 has been relatively well characterized among them. Arl1 is localized to the Golgi complex and recruits a subset of golgins onto Golgi membranes through binding directly to the GRIP domain at their C-termini. Mammalian ARFRP1 (ADP-ribosylation factor (ARF)-related protein 1), previously designated ARP is a membrane-associated 25 kDa GTPase with 33% of amino acids identical to those of ARF1. Arl3p, a yeast counterpart, shares 43% of amino acids with mammalian ARFRP1. Studies in yeast have shown that Arl3p is required for recruitment of Arl1p and the GRIP domain-containing protein, Imh1p/Sys3p, onto the Golgi. The functional importance of ARFRP1 is underscored by the finding that targeted disruption of the *Arfrp1* gene in mice resulted in embryonic lethality at gastrulation stage and apoptosis of ectodermal cells. However, the function of mammalian ARFRP1 has not been explored at the molecular level. Here, we demonstrate that ARFRP1 is associated mainly with the *trans*-Golgi compartment and the *trans*-Golgi network (TGN)

and is an essential regulatory factor for targeting of Arl1 and GRIP domain-containing proteins, golgin-97 and golgin-245, onto Golgi membranes. Furthermore, we show that ARFRP1 is involved in the anterograde transport from the Golgi to the plasma membrane as well as in the retrograde transport from endosomes to the Golgi.

1908

**The UBX Containing Protein UBXD4 Regulates Cell Surface Number and Subunit Stability of Alpha3-containing Nicotinic Acethylcholine Receptors**

K. Rezvani,[1] Y. Teng,[1] Y. Pan,[1] J. Cotton,[1] J. M. Lindstrom,[2] J. Dani,[1] M. De Biasi[1]; [1]Neuroscience, Baylor College of Medicine, Houston, TX, [2]Neuroscience, University of Pennsylvania Medical School, Philadelphia, PA

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels expressed in many regions of the central and peripheral nervous systems. nAChRs are necessary for proper synaptic transmission in ganglia and the number of functional nAChRs in neuronal membranes is a crucial factor for the efficacy of neurotransmission. By using the large cytoplasmic domain of α3 as a bait in the yeast two-hybrid system, we isolated a novel UBX-containing protein as the first cytosolic protein known to interact directly with the α3 nAChR subunit. The isolated clone was identified as protein UBXD4 (Accession number# NM_145441) which according to its role on nAChRs, we re-named "Nicotinic Receptor Partner Protein" or NRPP. Transient expression of tagged NRPP in HEK cells stably expressing α3* nAChRs facilitated cell surface expression of α3* receptors. The same effect was observed when NRPP was stably expressed in PC12 cells. Further experiments indicated that NRPP is located in the Golgi lumen, and that upon ubiquitination it is targeted by the 26S proteasome for degradation. Taken together our data suggests that NRPP may modulate the number of cell surface α3* nAChRs by controlling the stability of α3 and its distribution between specialized intracellular compartments and the plasma membrane. Key words: nicotinic acetylcholine receptor, α3 subunit, ubiquitin, proteasome, UBX domain, trafficking This work was supported by NIDA grants DA12661 and DA 017173 and a Philip Morris grant to MDB.

1909

**Function of the Phosphatidylinositol 4-Kinase Pik1p in a Golgi-to-Endosome Pathway Involving Gga2p**

L. Demmel,[1] M. Gravert,[1] E. Ercan,[1] M. Peter,[2] C. Walch-Solimena[1]; [1]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, [2]Institute of Biochemistry, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland

In *Saccharomyces cerevisiae*, experiments with temperature-sensitive mutants of the Phosphatidylinositol 4-kinase Pik1p revealed that the PI4P pool generated by this enzyme is essential for Golgi morphology, transport of cargo for exocytosis (invertase) as well as for vacuolar delivery (Carboxypeptidase Y, CPY). Moreover, several lines of evidence indicate that the PI4P pool at the Golgi represents a regulatory signal on its own. However, it remains elusive which proteins bind to PI4P and mediate its effects on Golgi function. In a screen for synthetic genetic interactions between a library of knockout of genes and a conditional-lethal *pik1* mutant allele, Gga2p has been isolated. As a role of Pik1p in control of vesicle formation at the Golgi has been suggested by previous work, we further investigated a possible common role of Pik1p and Gga2p. Gga proteins (Gga1p and Gga2p) are monomeric clathrin adaptors regulating Golgi-to-endosome transport. We provide data indicating that Pik1p, similar to Gga proteins, is involved in exit from the Golgi. Genetic and biochemical results were obtained suggesting that PI4P generated by Pik1p is required for normal Gga2p localization to the Golgi and for transport of cargo from the Golgi to the late endosomes as well as for a Golgi-to-surface pathway transiting an endosomal compartment. This proposed pathway is parallel to AP-1 mediated trafficking between Golgi and early endosomes. Thus, Gga2p might be a target of PI4P signaling at the Golgi and we further provide evidence for the existence of biosynthetic transport to endosomes in a specialized route for exocytosis.

1910

**Lipid Phosphatases Establish Spatially and Functionally Distinct Regions within the Golgi**

F. Cheong, B. Brankatschk, P. Mayinger; Department of Medicine, Division of Nephrology and Hypertension, Oregon Health and Science University, Portland, OR

Objective: To study the regulation of phosphoinositides at Golgi membranes by lipid phosphatases SAC1 and OCRL1. Methods: Vector-based RNA interference (RNAi) was used to deplete cellular levels of SAC1 and OCRL1. Confocal immunofluorescence microscopy was used to localize proteins in tissue culture cells. Bulk anterograde trafficking was monitored by pulse-labeled cells with 35S-Methionine. High performance liquid chromatography (HPLC) was employed to analyze phosphoinositides levels after in vivo labeling wih 3H-myo-inositol. Results: Phosphoinositides (PI) are important in regulating Golgi trafficking and structure. Phosphatidylinositol-4-phosphate (PtdIns(4)P) is required for anterograde carrier formation while phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) is responsible for maintaining the structural integrity of the Golgi. These phospholipids are tightly regulated by distinct phosphoinositides kinases and phosphatases. SAC1 is the main PtdIns 4-phosphatase at Golgi membranes while OCRL1 is a PtdIns(4,5)P2-specific 5-phosphatase. We found that SAC1 establishes low PtdIns(4)P regions in the Golgi. Golgi resident enzymes such as mannosidase II are concentrated in theses regions while cargo proteins are largely excluded. Depletion of SAC1 abrogated Golgi retention of mannosidase II and led to surface localization of this enzyme. In addition, Golgi morphology is dramatically affected in SAC1 depleted cells. In contrast, OCRL1 co-localizes with cargo proteins and knock down of this phosphatase caused defects in bulk anterograde trafficking. Conclusions: SAC1 and OCRL1 establish distinct phosphoinositide-specific regions within the Golgi that are instrumental for segregating anterograde trafficking from the recycling of resident Golgi enzymes.

1911

**Effect of a Chronic Deficiency of a $PIP_2$ 5-phosphatase on Cell Signaling**

S. F. Suchy, J. Cronin, R. L. Nussbaum; NHGRI/GDRB, NIH, Bethesda, MD

The importance of PI kinases and phospholipase C in regulation of $PIP_2$ levels in cell signaling is well established. However, the role of $PIP_2$ phosphatases in cell signaling has received little attention. This study was done to determine whether chronic deficiency of a $PIP_2$ 5-phosphatase, ocrl1, plays a role in calcium signaling. Ocrl1 is a ubiquitiously expressed, TGN/endosome localized enzyme and its deficiency in humans causes Lowe syndrome, characterized by bilateral congenital cataracts, renal Fanconi syndrome and mental retardation. Lowe patient fibroblasts were previously shown to have increased $PIP_2$ levels. In this study cell permeant AM esters of the $Ca^{2+}$ indicator dyes Fluo-4 and Fura red were used to

monitor changes in intracellular [$Ca^{2+}$] after agonist stimulation in patient and control fibroblasts. Several agonists were studied, including bradykinin, histamine and PDGF. Only the response to bradykinin differed consistently between Lowe and control fibroblasts. In 7 pairs of cell lines tested, Lowe cells had a peak [$Ca^{2+}$] after stimulation that was 25% higher than controls and reached their peak [$Ca^{2+}$] 20% faster than did controls. 2-Aminoethoxy-diphenylborate, an inhibitor of $IP_3$ induced calcium release, reduced the number of cells that responded to bradykinin in a dose dependent manner. There was no difference in total calcium stores, monitored by treatment with the ionophore A23187. Although the total bradykinin receptor concentration, measured by western analysis, did not differ between Lowe and control fibroblasts, Lowe cells had approximately two-fold more bradykinin receptors at the cell surface than controls, as measured in radioactive binding assays. This implies a decrease in receptor desensitization in Lowe cells. These findings indicate that although ocrl1 is concentrated at the TGN/endosome it plays a role in various functions at the plasma membrane including cell signaling and receptor desensitization.

1912

**Drs2p-Dependent Translocation of Phosphatidylserine and Phosphatidylinositol Fluorescent Analogs Across the Yeast Late Golgi Membrane**

P. Natarajan, T. R. Graham; Biological Sciences, Vanderbilt University, Nashville, TN

Phospholipid translocases (PLTs) are capable of flipping fluorescent or spin-labeled derivatives of phospholipids from the external leaflet of a membrane bilayer to the cytosolic leaflet and are thought to establish phospholipid asymmetry in biological membranes. The best candidates for PLTs are the Drs2p/ATPase II (ATP8A1) subfamily of P-type ATPases. Drs2p from budding yeast localizes to the *trans*-Golgi network (TGN) and is required for a flippase activity in this membrane. Using a Drs2 temperature-sensitive mutant, we have shown that ATP hydrolysis by Drs2p catalyzes the translocation of 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD) phosphatidylserine (PS) from the luminal leaflet to the cytosolic leaflet of this membrane. No Drs2p-dependent translocation of NBD-phosphatidylethanolamine or NBD-phosphatidylcholine was detected indicating headgroup specificity for the translocase. The specificity of Drs2p for NBD-PS suggested that translocation of PS would be required for the function of Drs2p in protein transport from the TGN. However, this is not the case, the PS-deficient *cho1* mutant does not phenocopy *drs2* but instead transports proteins normally through the secretory pathway. Moreover, a *drs2 cho1* double mutant retains *drs2* transport defects. Therefore, endogenous PS is not an essential substrate *in vivo* for the role Drs2p plays in protein transport. A search for additional substrates for Drs2p led to the surprising finding that Drs2p is required for the ATP-dependent translocation of bodipy-phosphatidylinositol (PI) and NBD-PI across the TGN membrane. These data suggest that Drs2p is a PS/PI translocase and further work is required to determine if PI translocation by Drs2p is needed to bud vesicles from the TGN.

1913

**Isolation of Extragenic Suppressors of the drs2 Cold-Sensitive Growth Defect in Saccharomyces cerevisiae**

B. Muthusamy, T. R. Graham; Biological Sciences, Vanderbilt University, Nashville, TN

Drs2p is an integral membrane P-type ATPase required for a phospholipid translocase activity in the *trans*-Golgi network (TGN) of *Saccharomyces cerevisiae.* The ATPase activity of Drs2p, and presumably its ability to flip phospholipid from the luminal leaflet to the cytosolic leaflet of the TGN, is required along with clathrin for formation of one of the two classes of post-Golgi, exocytic vesicles found in yeast. Disruption of *DRS2* yields a viable strain that grows well at 30C but fails to grow at 20C or below. Growth of *drs2Δ* at 30C requires the presence of three closely related P-type ATPases (Dnf1p, Dnf2p and Dnf3p) that show some functional redundancy with Drs2p. However, Drs2p provides an essential function at low temperatures that cannot be replaced by the Dnf ATPases. To better understand this essential function of Drs2p at low temperatures, we have screened for spontaneous bypass suppressors of the *drs2Δ* cold-sensitive growth defect. *drs2Δ* strains of opposite mating types were spread on rich medium at 17°C or 20°C and 19 spontaneous mutants were isolated that suppress the cold sensitive (cs) growth phenotype of *drs2Δ.* Genetic analyses of 19 cold-resistant *drs2Δ* strains indicated that 3 dominant and 15 recessive suppressors were caused by single gene mutations, and one recessive mutant was caused by multiple mutations. The 15 recessive mutants fell into two complementation groups called *sdk1* (12 alleles) and *sdk2* (3 alleles) for **s**uppressor of ***d****rs2* **k**nockout. We are in the process of cloning *SDK1* and *SDK2* by transforming the recessive suppressor strains with a genomic library and screening for complementation of the cold-resistant growth phenotype.

1914

**The Role of Sphingomyelin Synthase-Derived Diacylglycerol on Vesicular Transport**

Y. Ueda,[1] S. Yamaoka,[2] T. Okazaki,[2] T. Kobayashi[1]; [1]Discovery Research Institute, RIKEN, Wako, Japan, [2]Pathophysiological and Therapeutic Science, Tottori University Faculty of Medicine, Yonago, Japan

Sphingomyelin is a major cell surface sphingolipid. Sphingomyelin synthase is proposed to locate in the Golgi apparatus. During the lipid synthesis, phosphocholine moiety of phosphatidylcholine is transferred to ceramide. Therefore, sphingomyelin systhesis is accompanied by the formation of diacylglycerol. The role of this diacylglycerol has long been discussed. Fumonisin B1 inhibits ceramide synthesis and thus prevents sphingomyelin synthase-derived diacylglycerol production. Using fumonisin B1, it has been suggested that the diacylglycerol formed during sphingomyelin synthesis is involved in vesicular traffic from the Golgi apparatus. In addition to the inhibition of diacylglycerol synthesis, fumonisin B1 accumulates sphingoid bases that are growth inhibitory and pro-apoptotic for many types of cells. In order to directly assess the effect of the sphingomyelin synthesis dependent diacylglycerol on membrane traffic, we employed recently isolated sphingomyelin synthase defective mouse lymphoid cell mutants. Whereas the mutants contain less diacylglycerol than sphingomyelin-positive revertant cells, the release of glucosaminoglycan was not significantly altered between two cell types. Addition of fumonisin B1 decreased the release of glucosaminoglycan in both mutants and revertants. These results suggest that sphingomyelin synthase-derived diacylglycerol is not involved in the secretion of glucosaminoglycan and fumonisin B1 inhibits vesicular traffic via sphingomelin-independent mechanism.

1915

**Discrete Domains within Naked2 Cooperate in the Transport of TGF-α-containing Exocytic Vesicles to the Basolateral Surface of Polarized Epithelial Cells**

C. Li, M. Hao, Z. Cao, W. Ding, R. Graves-Deal, J. Hu, D. W. Piston, H. A. Brown, R. J. Coffey; Departments of Medicine, Pathology, and Cell

and Developmental Biology, Vanderbilt University Medical Center and Dept of Veterans Affairs Medical Center, Nashville, TN

Mechanisms underlying basolateral transport of exocytic vesicles to the plasma membrane are largely unresolved. We previously reported that Naked2 associates with exocytic vesicles containing a Golgi-processed form of TGFα and escorts TGFα to the basolateral surface of polarized MDCK cells in a Naked2 myristoylation-dependent manner (PNAS 101: 5571-5576, 2004). Here we show that residues 51-173 of human Naked2 provide basolateral directionality in that fusion of these residues to a $Na^+/H^+$ exchanger regulatory factor 1 (NHERF1)-DsRed2 fusion construct completely redirects fluorescence from the apical cytoplasm to the basolateral plasma membrane of MDCK cells. Residues 1-36 bind PIPn broadly, and, in the absence of N-myristate, confer general post-Golgi vesicle recognition. Myristoylation of Naked2 is required for docking and fusion of Naked2 vesicles, but not for vesicle recognition or trafficking of these vesicles in the basolateral cytoplasm. In μ-1B deficient polarized LLC-$PK_1$ cells, both Naked2 and TGFα traffic normally to the basolateral membrane. These data demonstrate that Naked2 is an adaptor-like protein that contains distinct functional domains that orchestrate the delivery and fusion of exocytic vesicles at the basolateral plasma membrane of polarized epithelial cells in a μ-1B-independent manner.

1916

### Purification and Characterization of Naked2-Associated Exocytic Vesicles

Z. Cao,[1] C. Li,[1] R. Graves-Deal,[1] A. L. Ham,[2] J. N. Higginbotham,[3] J. L. Franklin,[1] R. J. Coffey[1]; [1]Departments of Medicine, Cell and Developmental Biology, Vanderbilt University, Nashville, TN, [2]Mass Spectrometry Research Center, Vanderbilt University, Nashville, TN, [3]Departments of Pediatric Infectious Diseases, Vanderbilt University, Nashville, TN

Genetic studies in *Drosophila* have established that activation of canonical Wnt signaling results in upregulation of Naked Cuticle that then binds and inactivates Dishevelled, a positive regulator of Wnt signaling. Two mammalian Naked homologs, Naked 1 and 2, have been found. We recently reported that Naked2, but not Naked1, interacts with the cytoplasmic tail of pro-TGFα, and escorts post-Golgi TGFα-containing exocytic vesicles to the basolateral surface of polarized epithelial cells, where these vesicles dock and fuse in a Naked2 myristoylation-dependent manner. In GFP-tagged, myristoylation-deficient Naked2-expressing polarized MDCK cells (G2A Naked2-GFP), Naked2-associated vesicles are trapped at the basolateral corner and TGFα is unable to reach the basolateral membrane (PNAS 101:5571-5576, 2004). We have successfully isolated G2A Naked2-GFP-containing vesicles using a combination of iodixanol density gradient separation and dual color labeling flow sorting (DiD:1,1'-dioctadecyl-3,3,3',3'-tetramethylindodi-carbocyanine perchlorate and GFP) by using BD FACSAria flow cytometry. Sufficient amounts of these flow cytometric-enriched vesicles have been isolated to perform proteomic analysis by LC MS/MS. The identification of proteins that comprise the machinery and additional cargo of this specialized subset of exocytic vesicles will be presented.

1917

### Identification of Signals in ATP7B Mediating Its Cu-dependent Trafficking in Polarized Hepatic Cells

Y. Guo, L. K. Nyasae, L. T. Braiterman, A. L. Hubbard; Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

Copper (Cu) is an essential cofactor of cellular proteins but is toxic in its free state. The hepatic Cu-ATPase, ATP7B, has two functions in Cu homeostasis: it loads $Cu^+$ onto newly-synthesized apo-ceruloplasmin in the secretory pathway thereby activating the plasma protein; and it participates in the excretion of excess $Cu^+$ into the bile. To carry out these two functions, the membrane protein responds to changes in intracellular Cu levels by cycling between the Golgi and apical region. Using polarized hepatic WIF-B cells and confocal microscopy, we found that both endogenous and exogenous ATP7B resided in the Golgi region in Cu-depleted cells, while in Cu-loaded cells, the protein relocated to unique vesicles near to the apical plasma membrane as well as the membrane itself. To determine the roles of ATP7B's cytoplasmic N- and C-termini in regulating its trafficking, we generated 9 mutation/deletions and analyzed their behaviors in WIF-B cells. Truncation of the ATP7B N-terminus up to the fifth copper binding domain yielded an active ATPase that was insensitive to cellular Cu levels and constitutively trafficked to the opposite (basolateral) plasma membrane domain. Fusion of the N-terminal 63aa of ATP7B to the truncated protein restored both its Cu-responsiveness and correct intracellular targeting, indicating that important targeting information is contained in this sequence. We also identified two signals, tri-L (L1454-1456) and di-E (E1461-1462), in the C-terminus of ATP7B. In Cu-loaded WIF-B cells, the tri-L mutant traffics to apical vesicles and the apical membrane, while the di-E protein traffics to the vesicles only. When Cu levels are decreased, neither mutant protein returns to the TGN region, suggesting that the two motifs contain retrieval signals. We are currently searching for binding partners.

1918

### Adenosine Receptor-dependent Modulation of Bladder Umbrella Cell Surface Area

W. Yu, G. L. Apodaca; Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA

Adenosine receptors are expressed in all tissues and play important roles in regulating cellular functions such as neurotransmission, cell polarization, and exocytosis. Bladder umbrella cells undergo cycles of exocytosis/endocytosis as the bladder fills and empties. Previous data showed that modulation of umbrella cell surface area occurs through a P2X purinergic receptor-, cAMP- and PKA- dependant mechanism. To test the hypothesis that adenosine receptors are also involved in this process, isolated uroepithelium was mounted in modified Ussing chambers and changes in membrane capacitance (where 1uF=1cm2 surface area) were measured in the presence of adenosine or different adenosine receptor agonists. Administration of adenosine to the serosal or mucosal surface of uroepithelium led to increased membrane capacitance of ~30% or ~24%, respectively, after 5 hours, and the administration of deaminase abolished adenosine-induced capacitance increases. While A1, A2a and A3 agonists (CCPA, CGS21680, IB-MECA, respectively) all increased the membrane capacitance gradually after being administrated serosally (~22%, ~33%, ~26%), only A1 agonist increased the membrane capacitance significantly after being administrated mucosally (~39%). Adenosine receptor antagonists, as well as deaminase, had no effect on pressure-induced capacitance increases. 2-APB or calcium-free Krebs solution inhibited adenosine- or agonists- induced capacitance increases. Western blot analysis confirmed the expression of all 4 receptors in uroepithelium, and immunofluorescence staining also showed that A1, A2a, A2b and A3 receptors were expressed in the uroepithelium/submucosa. Upon stretch, adenosine was released significantly to both mucosal and serosal chamber measured by HPLC. These results indicate that adenosine receptor signaling may contribute to the modulation of bladder umbrella cell exocytosis through a calcium dependent pathway.

1919

**Crn7 is Crucial for the Golgi Complex Architecture and Function**

V. Rybakin,[1] N. Gounko,[2] R. Müller,[1] A. A. Noegel[1]; [1]Biochemistry I, University of Cologne, Cologne, Germany, [2]Cell Biology, University of Groningen, Groningen, The Netherlands

Crn7 is a novel mammalian WD-repeat protein. Using biochemical methods and microscopy, we established that Crn7 is present at the cytosolic side of early Golgi membranes, and in the cytosol. Golgi localization of Crn7 was further shown to depend on the integrity of the ER-to-Golgi transport and on tyrosine phosphorylation of Crn7. The protein is still present on ER-Golgi chimaeric membranes formed upon the BFA treatment, suggesting that the COPI system or preservation of the Golgi membrane identity is not important for its localization. Using Crn7 siRNA, we established that the protein is indeed important for the Golgi structure and function. The structure of the Golgi matrix is significantly altered in RNAi cells. GRASP65 displays scattered staining pattern in dot-like structures. The bulk of GM130, a protein depending on GRASP65 in its matrix localization, is displaced to the cytosol, and only a minor amount remains in the matrix remnants. These data suggest that GRASP65 alone is not sufficient to drive the matrix localization of GM130. The trans-Golgi network is disassembled into smaller flat cisternae in Crn7 RNAi cells, as shown by TGN38 staining. This is apparently not a direct effect of the Golgi matrix disassembly, as the matrix is predominantly present in the cis-zone of the Golgi complex, as shown by previous studies. Thus, Crn7 knockdown results in profound defects in the Golgi architecture. These defects correlate with functional abnormalities. TGN38 protein, known to cycle between the TGN and cell surface, redistributes from scattered trans-Golgi remnants to cytoplasmic vesicles, implying that proteins destined to downstream locations are gradually depleting from the Golgi, while there is no import of new cargo. This is further confirmed by the fact that lysosomal LAMP1 staining is depleted in cells stably expressing Crn7 siRNA.

1920

**A Peripheral Membrane Multi-protein Complex that Binds to Chitin Synthase III is Important for its Transport from the Trans-Golgi Network to the Cell Surface**

S. Sanchatjate, R. Schekman; University of California, Berkeley, Berkeley, CA

In *Saccharomyces cerevisiae*, the plasma membrane protein chitin synthase III (Chs3p) synthesizes chitin to strengthen the yeast cell wall. Unlike most plasma membrane proteins whose transport to the cell surface is tightly linked to gene expression and protein synthesis, Chs3p follows a unique itinerary. In steady state, Chs3p resides in internal punctate structures of trans-Golgi network (TGN) and early endosome. During the cell cycle or upon heat shock, Chs3p is rapidly mobilized to the cell surface and subject to rounds of internalization and re-export. Chs5p and Chs6p are two peripheral membrane proteins necessary for the transport of Chs3p from the TGN to the plasma membrane. We have found that Chs5p forms large stable complexes with Chs6p and three Chs6 paralogs: Ymr237w, Ykr027w, and Bud7p. These Chs5p/Chs6p complexes transiently interact with its cargo, Chs3p, and the presence of Chs3p in the complexes is dependent on every subunit. Chs5p and Chs6p have unique and crucial roles in Chs3p transport because either *chs5Δ* or *chs6Δ* mutant drastically reduces the level of Chs3p bound to the remaining subunits of the complex. Ymr237w and Bud7p appear to have redundant function in Chs3p transport because double deletion of both is necessary to displace Chs3p from the complex. Our results suggest that these Chs5p/Chs6p complexes may function as adaptor and possibly coat protein complexes which selectively transport certain cargos from the TGN to the plasma membrane in response to regulatory cues (see poster by R. Barfield).

1921

**Chs5p, Bud7p, and YMR237w Localize Fus1p to the Plasma Membrane in *Saccharomyces Cerevisiae***

R. M. Barfield, R. Schekman; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

During *Saccharomyces cerevisiae* mating, the single-pass transmembrane protein Fus1p is transported to the plasma membrane by a mechanism requiring the peripheral membrane protein chitin synthase 5 (Chs5p) (1,2). In the absence of Chs5p, Fus1p no longer localizes to the plasma membrane but instead localizes to intracellular punctate structures, probably representing a late golgi compartment (2). We know that Chs5p functions with Chs6p to transport the cargo molecule Chs3p to the plasma membrane (3). We also know that Chs5p forms a complex *in vivo* with Chs3p, Chs6p, and three Chs6p-related proteins, YKR027w, Bud7, and YMR237w (see poster by S. Sanchatjate). Therefore, we hypothesize that Fus1p forms a complex with Chs5p and a Chs6p-like protein(s) and that transport of Fus1p to the plasma membrane requires a Chs6-like protein(s). We have found that Fus1p co-purifies with Chs5p *in vivo* and that the intracellular domain of Fus1p interacts with purified Chs5p *in vitro*. We have also found that the intracellular domain of Fus1p interacts with YMR237w and YKR027w in a yeast two-hybrid assay. Using fluorescence microscopy and a protease protection assay, we have analyzed Fus1p plasma membrane localization in cells mutant for one or more *CHS6*-related gene. Whereas single mutants have a wild-type Fus1p localization pattern, *bud7 YMR237w* double mutants are defective in Fus1p plasma membrane localization. Based on our biochemical and genetic data, we propose a model where Chs5p and Bud7p/YMR237w recruit Fus1p into a complex to transport Fus1p to the plasma membrane. 1. Trueheart *et al*. (1987) *Mol Cell Biol* 7, 2316-2328. 2. Santos and Snyder. (2003) *Eukaryotic Cell* 4, 821-25. 3. Valdivia *et al*. (2002) *Dev Cell* 2, 283-94.

1922

**Clathrin Participates in the Exit of a Subset of Cargo Proteins from the TGN**

S. Deborde, E. Perret, D. Gravotta, E. Rodriguez-Boulan; Ophthalmology, Weill Medical College of Cornell University, New York, NY

Clathrin coated vesicles are present not only at the plasma membrane but also at the trans Golgi network (TGN), considered a major segregation site for apical and basolateral proteins in polarized cells. Whereas the function of clathrin in the endocytic process has been extensively studied, its function at the TGN remains to be demonstrated. We disrupted the function of clathrin using MDCK cells stably transfected with FKBP-light chain a, which can be reversibly cross-linked with a permeable bifunctional reagent (Moskowitz et al., Mol Biol Cell, 2003). We first showed that the maturation of the lysosomal hydrolase cathepsin D was impaired upon addition of the cross-linker. We then investigated the role of clathrin on the exit from the Golgi complex of GFP-tagged basolateral membrane proteins NCAM and VSVG and apical membrane protein p75-NTR. MDCK cells were microinjected in the nucleus with cDNAs encoding the membrane proteins, which were accumulated in the TGN using a 20°C temperature block. We then measured the exit of the GFP-tagged protein from the Golgi at 32°C in presence or absence of the cross-linker. The cross-linker did not modify the exit of p75-GFP or VSVG-GFP, whereas it delayed the exit of NCAM. The half time of TGN emptying was

increased from 40 to 100 min. These experiments clearly demonstrate a role of clathrin at the TGN level and this effect is protein-specific.

1923

### GGA Function is Required for Membrane Remodeling and Substrate Processing in Maturing Neuroendocrine Secretory Granules

O. Kakhlon,[1] P. Sakya,[2] B. Larijani,[2] S. A. Tooze[1]; [1]Secretory Pathways Laboratory, Cancer Research UK, London, United Kingdom, [2]Cell Biophysics Laboratory, Cancer Research UK, London, United Kingdom

The recently described clathrin adaptor GGA (Golgi associated, γ-ear containing, ARF (ADP-ribosylation factor) binding protein) has been implicated in sorting of Mannose-6-Phosphate receptors (MPRs) from the trans-Golgi network (TGN) to endosomes. As secretory granule (SG) maturation has been proposed to involve formation of clathrin-coated vesicles (CCVs) from immature SGs (ISGs), we test here the effect of repression of GGA function on maturation of SGs in neuroendocrine cells. Overexpression of a truncated GFP tagged GGA, VHS-GAT-GFP, containing only the cargo (VHS) and ARF (GAT) interacting domains, led to retention of the SNAREs VAMP 4 and syntaxin 6 in mature SGs (MSGs). This suggested that membrane remodelling by ISG-derived CCV budding is down-regulated by the SG-localizing VHS-GAT-GFP. Furthermore, our flow cytometry data demonstrate that in VHS-GAT-GFP-expressing cells, Secretogranin II (SgII) processing in SGs is inhibited, and the positive correlation between the levels of the prohormone convertase 2 (PC2) and the SgII processing product p18 is abrogated. All these effects were not observed if full-length GGA1-GFP was overexpressed. Neither GGA1-GFP, nor VHS-GAT-GFP perturbed SG protein sorting in, or budding from the TGN. A new fluorescence-based ISG homotypic fusion assay demonstrates that repression of GGA function also does not affect homotypic fusion of ISGs. Lastly, reducing GGA3 levels by short interfering (si) RNA also down-regulated SgII processing by PC2. Our results suggest that when GGA activity is repressed SgII processing is compromised due to a specific inhibition of ISG-derived CCV budding. We propose that formation of CCVs from maturing SGs is required for the maintenance of SG function.

1924

### Dynamic Actin has Roles in Trans-Golgi Network Tubulation and Post-Golgi Carrier Budding

L. A. Hammond, J. L. Stow; Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD, Australia

Post-Golgi carriers (PGC) bud from the trans-Golgi network (TGN) by a process of membrane tubulation. Proteins of the GRIP-domain golgin family selectively associate with these membrane tubules during this process and their GFP-tagged GRIP-domains serve as markers to study such events. Microtubules and a dynamic population of short actin microfilaments have been observed to associate with the TGN and with budding PGCs. Here we have examined the role of actin filaments and drug effects in membrane tubulation using real time epifluoresence and 4-dimensional confocal imaging. Cytochalasin D, latrunculin A and jasplakinolide all resulted in significant perturbation of membrane tubulation and PGC budding. The effect of actin targeting agents does not alter the velocities of tubule extension, which instead was shown to be a microtubule dependent event. In accordance with these findings, a close association between microtubules and short, dynamic actin microfilaments has been demonstrated. It is proposed that dynamic actin plays a role in the preliminary stages of PGC bud formation. However, once an initial bud has formed the emerging tubule becomes microtubule dependent to be drawn out and eventually detach.

## Endocytic Machinery: Structure, Function & Regulation (1925-1949)

1925

### Regulation of the Ubiquitin Ligase Itch by the Epidermal Growth Factor Receptor (EGFR)

B. A. Azakir, A. Angers; Biological Sciences, University of Montreal, Montreal, PQ, Canada

Endophilin is an endocytic protein first identified because of it Src homology 3 (SH3) domain and its interaction with dynamin and synaptojanin. Endophilin A1 promotes the epidermal growth factor receptor (EGFR) internalisation in a tripartite complex with cbl and CIN-85. Recently, we showed that endophilin A1 is a substrate for Itch, a HECT domain ubiquitin ligase. Upon activation of EGFR by EGF, Endophilin is translocated to endosomes where it colocalizes with Itch. Here, we show that like Endophilin, Itch itself is ubiquitinated upon stimulation with EGF. EGF-induced ubiquitination of both proteins is transient, but does not lead to proteasomal degradation as the proteasome inhibitor Lactacystin has no effect. Recently, it has been reported by others that the activated stress kinase JNK can phosphorylate Itch and increase its ubiquitin ligase activity. JNK can also be activated by EGF treatment. Endophilin is also involved in another JNK activation pathway through its binding to the Map 4 kinase GLK. We thus examined the effect of GLK and Endophilin overexpression on Itch autoubiquitination. We found that GLK activity led to increased Itch ubiquitination, and that Endophilin A1 could regulate the effect. Together, these data suggest that Itch activity can be regulated by endocytic processes involving Endophilin, tyrosine kinase receptors, and the JNK pathway.

1926

### The Ubiquitin Ligase Itch Binds and Is Regulated by the Ubiquitin Protease FAM

R. Moutchantaf,[1] B. Azakir,[1] P. S. McPherson,[2] S. A. Wood,[3] A. Angers[1]; [1]Biological Sciences, Montreal University, Montreal, PQ, Canada, [2]Neurology and Neurosurgery, Montreal Neurological Institute, Montreal, PQ, Canada, [3]Child Health Research Institute, North Adelaide, Australia

Itch is an ubiquitin ligase closely related to Nedd4, a HECT domain ligase implicated in the regulation of epithelial sodium channels by endocytosis. We have identified Itch as a binding partner of the endocytic accessory protein endophilin, and found it to be localized to endosomes and the trans-Golgi network (TGN) in mammalian cells. Using a GST-Itch affinity column and tandem mass spectrometry, we identified the ubiquitin-protease FAM as an Itch-binding partner. FAM (also known as Usp9x) is the mammalian homologue of *faf* in *Drosophila*, known to regulate the expression of the *Drosophila* epsin homologue and to thereby play a regulatory role in endocytosis. FAM also localizes to endosomes and the TGN where it overlaps significantly with Itch. We mapped the FAM-binding domain on Itch to the WW domains, small protein interaction modules usually involved in substrate recognition. However, upon co-expression of both proteins with ubiquitin in HEK-293T cells, we found that FAM is not ubiquitinated by Itch. Rather, Itch is readily auto-ubiquitinated, and this ubiquitination is fully reversed by the activity of FAM. We thus conclude that Itch is a substrate for the de-ubiquitinating activity of FAM. This observation suggests that FAM activity could stabilize the level of Itch protein in cells. Accordingly, we found that overexpression of FAM led to the accumulation of Itch in transfected cells. Likewise, expression levels of the proteins parallel each other in a variety of cell lines. We suggest that Itch is a functional substrate of FAM, and that this

interaction is important to regulate Itch and processes in which Itch has been identified as the ligase.

1927

**UIM-dependent Regulation of the Yeast Hrs Homologue, Vps27**

W. W. Lin, R. Dunn, R. Hostetler, L. Hicke; Biochemistry Molecular Biology & Cell Biology, Northwestern University, Evanston, IL

Endocytosis is a cellular process that regulates proteins involved in communication between the cell and its environment. Ubiquitin is a short polypeptide that can be covalently conjugated onto substrates to modify protein conformation, localization, and/or function. Ubiquitin plays a key role in multiple steps of endocytosis. It regulates receptor internalization at the plasma membrane and sorting at multivesicular endosomes (MVE) by acting as a cargo localization signal and regulating activity of endocytic machinery. These ubiquitin signals are recognized by ubiquitin-binding domains (UBD) that have been identified in many proteins along the endocytic pathway. In addition to recognizing the ubiquitin moiety in *trans*, ubiquitin-binding also promotes monoubiquitination in *cis*. The relationship between ubiquitin-binding by and ubiquitination of endocytic proteins has yet to be understood. In this study, we analyze this relationship by investigating how Vps27 ubiquitination is regulated and how monoubiquitination affects Vps27 function. Vps27 is an ubiquitin-binding vacuolar protein sorting (VPS) protein that is essential for cargo sorting at the MVE. Vps27 binds ubiquitin via two ubiquitin-interacting motifs (UIM). Vps27 is monoubiquitinated by the ubiquitin ligase Rsp5 in an uncharacteristic manner that is independent of Rsp5 C2 and WW domains. Ubiquitination of Vps27 is dependent on its UIMs and probably occurs on a series of lysines immediately N-terminal to the UIMs. Another UBD, the UBA domain, does not efficiently mediate ubiquitination of Vps27, but does replace ubiquitin-binding and rescues growth phenotypes of *vps27*-UIM mutants. Vps27 is also phosphorylated, and this modification does not require the UIMs. These findings suggest that while ubiquitination is dependent on the UIMs, the role of the UIMs in Vps27 function is predominantly in binding ubiquitin. Further characterization of the Vps27 UIMs and the lysine mutants will define the role of Vps27 ubiquitin-binding and how ubiquitin-binding is regulated.

1928

**Role of Hrs UIM-mediated Ubiquitin Signaling in the Coordination of Multiple Steps in Endosomal Trafficking**

J. W. Pridgeon, J. A. Olzmann, E. A. Kirk, L. Chin, L. Li; Department of Pharmacology, Emory University School of Medicine, Atlanta, GA

Ubiquitination plays a crucial role in the control of diverse cellular processes, and its defective regulation has been implicated in the pathogenesis of a variety of human diseases. However, the molecular mechanisms by which cells recognize and transmit ubiquitin signals remain poorly understood. The ubiquitin-interacting motif (UIM) is a conserved ubiquitin binding module found in many proteins, including hepatocyte growth factor regulated tyrosine kinase substrate (Hrs), a key component of the endosomal sorting machinery. Here we show that the Hrs UIM domain preferentially binds K48- and K63-linked polyubiquitin chains over monoubiquitin. By using an in vitro expression cloning (IVEC) screening approach, we have identified 47 ubiquitinated proteins that are specifically recognized by the UIM domain of Hrs. The validity and specificity of the IVEC screen have been confirmed by subsequent characterization of two identified proteins, Munc18-1 and Hsc70. Surprisingly, only a small fraction of the identified Hrs UIM-interacting ubiquitinated proteins are membrane cargo proteins. Major classes of the identified proteins include components of the vesicular trafficking machinery, cell signaling molecules, membrane protein-associated adaptor proteins, components of cytoskeleton and cytoskeleton-dependent transport, and enzymes involved in ubiquitination, lipid metabolism, and other metabolic processes. Furthermore, we have identified 6 novel proteins. Our results reveal the involvement of Hrs UIM-mediated ubiquitin signaling in the coordination of multiple steps in endosomal trafficking as well as in the regulation of cell signaling, cytoskeleton and membrane dynamics, and other cellular processes. Supported by NIH Grant NS047575

1929

**Ubiquitination of Hrs by a Novel UBE4B (E3) Ligase Involved in EGFR Degradation**

Q. Yan, W. Sun, Y. Lotfi, A. J. Bean; Neurobiology and Anatomy, University of Texas Health Science Center at Houston, Houston, TX

Altering the number of surface receptors can rapidly modulate cellular responses to extracellular signals. Some receptors, like the EGF receptor, are internalized after ligand binding and are transported to late endosomes/multivesicular bodies, where they either remain on the limiting membrane from where they may be recycled, or are sorted into luminal vesicles resulting in lysosomal degradation. This critical step in the sorting of internalized proteins on the limiting membrane of a multivesicular body is hypothesized to involve attachment and removal of ubiquitin sorting signals recognized by sorting complexes. We have observed a direct interaction between hrs, a protein that recruits both of the hypothesized sorting complexes to endosomal membranes, and an E3 ubiquitin ligase, UBE4B. Hrs recruits UBE4B to endosomal membranes where it binds saturably. UBE4B monoubiquitinates hrs both *in vitro* and *in situ* and a UBE4B mutant lacking the catalytic U-box acted in a dominant negative manner, inhibiting basal ubiquitination of hrs. Overexpression of UBE4B had no effect on the level of hrs protein compared to normal HeLa cells, nor were there alterations in the levels of other proteins that interact with either hrs or UBE4B such as actinin-4, Eps15, VCP, or Syntaxin13. These data suggest that the ubiquitination of hrs by UBE4B does not alter its stability. Overexpression of UBE4B or blocking the interaction of hrs and UBE4B by expressing a small fragment of hrs required for their binding affected EGFR degradation suggesting that the ubiquitination of hrs may affect the sorting machinery. Depletion of UBE4B also affected EGFR trafficking.

1930

**Hrs Interacts with the E3 Ubiquitin Ligase UBE4B**

Y. Lotfi, W. Sun, Q. Yan, A. J. Bean; Neurobiology and Anatomy, University of Texas Health Science Center at Houston, Houston, TX

The endocytic pathway can be separated into numerous stages based on the movement of cargo. Following transport from early to late endosomes, proteins to be degraded in the lysosome are internalized into the lumen of the late endosome via a process of membrane invagination and vesicle fission resulting in the formation of an organelle with a limiting membrane, (from which recycling occurs,) and intralumenal vesicles (that are degraded in lysosomes), called a multivesicular body (MVB). The sorting step at the limiting membrane of the MVB has been shown to involve ubiquitination. Hrs is an endosome-associated protein that functions to recruit proteins to the endosomal membrane that affect endosomal sorting/trafficking. Hrs interacts with a number of proteins including eps15, tsg101, SNAP-25 and STAM, all previously implicated in membrane sorting/trafficking. We have detected an additional interaction of hrs with the E3 ubiquitin ligase UBE4B. We identified the hrs-UBE4B interaction

using a two-hybrid screen and confirmed this interaction using immunoprecipitation and in vitro binding assays. UBE4B bound to hrs in the region between the FYVE domain and the coiled-coil region. We observed that when expressed alone, UBE4B is cytosolic and it is recruited to endosomal membranes when coexpressed with hrs, suggesting that hrs may recruit UBE4B to endosomes. In this regard, recombinant UBE4B bound saturably to endosomal membranes and this binding was blocked by addition of the fragment of hrs required for UBE4B binding. UBE4B is a member of the U-box protein family that mediates ubiquitination of various target proteins. We examined whether UBE4B might function to ubiquitinate hrs using in vivo and in vitro assays. We observed that UBE4B does ubiquitinate hrs. The effect of this ubiquitination on the function of hrs is under investigation.

1931

**HIM, a Novel Hrs-Interacting Molecule Implicated in Endosomal Trafficking**

E. A. Kirk, L. Chin, L. Li; Department of Pharmacology, Emory University School of Medicine, Atlanta, GA

Endocytic trafficking is crucial for cell development, immune response, and signal transduction. Internalized cell surface receptors are rapidly and efficiently sorted between recycling and degradative pathways. Despite recent progress in the identification of proteins involved in endocytic trafficking, the precise molecular mechanisms that govern sorting and transport of proteins to the lysosome remain poorly understood. Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) has recently been implicated as a key player in the regulation of endosomal cargo sorting and multivesicular body formation. In an effort to further understand the mechanism of action and regulation of Hrs, we performed a yeast two-hybrid screen to search for proteins that interact with Hrs. Here, we report the identification of a novel 105 kDa Hrs-interacting molecule named HIM. Unlike the ubiquitously expressed Hrs, HIM is detectable only in brain, heart, kidney, and testis, suggesting that HIM may be involved in tissue-specific regulation of Hrs function. We have confirmed the Hrs-HIM interaction by co-immunoprecipitation and mapped the binding domains of Hrs and HIM that mediate their association. Subcellular fractionation studies reveal the presence of HIM in membrane-associated pools and immunofluorescence confocal microscopic analyses demonstrate that HIM is partially co-localized with Hrs on early endosomes. Furthermore, overexpression of HIM inhibits ligand-induced degradation of the epidermal growth factor receptor (EGFR) without affecting either constitutive or ligand-induced receptor-mediated endocytosis. These results suggest that HIM is a functional partner of Hrs in the regulation of endosome-to-lysosome trafficking. This work is supported by NIH grant NS047575.

1932

**MTMR2 Interacts with hVPS34/p150 to Regulate Late Endosomal PI3P Formation**

C. Cao; Dept of Pathology, University of New Mexico Health Science Center, Albuquerque, NM

Phosphatidylinositol 3-phosphate (PI3P) plays a regulatory role in vesicular trafficking and cargo sorting by serving as a binding site for effector proteins containing phosphoinositide-binding domains. PI3P formation on late endosomes is initiated by active Rab7, which in turn regulates the activity of the hVPS34/p150 PI 3-kinase. The recruitment of myotubularin lipid phosphatases in response to localized PI3P synthesis serves to control endosomal PI3P levels. For proper late endosome function, the synthesis and degradation of PI3P must be tightly coordinated. Our data suggest a novel mechanism whereby the myotubularin lipid phosphatase related protein 2 (MTMR2) regulates late endosomal PI3P formation. Immunofluorescence staining shows that MTMR2 colocalizes with hVPS34 and Rab7. In vivo coimmunoprecipitation between MTMR2, hVPS34/p150 and rab7 demonstrate that both MTMR2 and Rab7 interact with p150 while they do not interact with each other. Furthermore, in vitro studies identify that an overlapping binding site consisting of the HEAT and WD40 domains of p150 mediates a competitive binding of MTMR2, or rab7, to p150. Our study suggests that MTMR2 regulates late endosomal PI3P formation through a competitive binding mechanism where Rab7 must be released from p150 to allow binding of MTMR2. Work supported by NSF MCB9982161 and UNMSOM.

1933

**PI3K-C2a Modulation of Clathrin Coated Membrane Dynamics**

Y. Zhao, J. H. Keen; Dept. of Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

Clathrin mediated membrane trafficking requires coordination of protein-protein and protein-lipid interactions in a precise spatial and temporal order. The Class II PI3K-C2α has recently been identified as a component of clathrin coated pits (CCPs) in cells, and its exogenous expression can both inhibit clathrin mediated membrane trafficking at the plasma membrane and trans-Golgi network, and induce the proliferation of novel, cytoplasmic clathrin coated structures (CCSs) (Gaidarov et al., *Molecular Cell*, 2001). Here we examine the interrelationship of PI3K-C2α expression and localization with the dynamics of clathrin coated membranes. siRNA-mediated knockdown of PI3K-C2α levels results in inhibition of endocytosis, confirming its involvement in the uptake pathway. At relatively low levels of exogenous expression, PI3K-C2α is enriched in forming CCPs in a stage-specific manner, and escorts endocytic cargos through endosomal compartments. At higher levels, PI3K-C2α is constitutively associated with clathrin, leading to stationary CCPs on the plasma membrane and highly motile, intracellular CCSs in the cytoplasm. The average speed of these CCSs is >10 times faster than that of canonical clathrin coated vesicles and endosomal buds. However, their rapid movement appears restricted to relatively short range, suggesting that they are novel, clathrin decorated structures. The PI3K-C2α-induced movement of intracellular CCSs is primarily microtubule-based, and assays reveal the existence of a complex between PI3K-C2α and dynactin subunits. These findings suggest that PI3K-C2α plays key roles in the coordination required for endocytic trafficking. By providing a structural link between clathrin coated membranes and the microtubule motor machinery, it is part of the molecular mechanism responsible for movement of plasma membrane and endosomal CCSs, and its lipid kinase activity is also likely to be functionally involved in modifying the local membrane environment of CCSs.

1934

**Characterization of Rab7 Interactions with Lipid Kinases and Phosphatases**

M. Tessema,[1] C. Cao,[1] M. Stein,[2] P. C. Simons,[1] L. A. Sklar,[1] A. Wandinger-Ness[1]; [1]Pathology, University of New Mexico, Albuquerque, NM, [2]Section of Microbial Pathogenesis, Yale University, New Haven, CT

Rab7 plays a pivotal role in endocytic transport, in part by regulating late endosomal phosphoinositide signaling and metabolism. GTP-bound Rab7 interacts with the human phosphatidyl inositol (PI) 3 kinase (hVPS34) and its adaptor protein (p150) and regulates the activity and function of the

hVPS34 kinase on late endosomes. The downstream lipid kinase, PIKfyve, also localizes on late endosomes and utilizes the hVPS34 product PI3P to produces PI3,5P. Late endosomal PI3P and PI3,5P are pivotal in recruiting trafficking cofactors and regulatory lipid phosphatases such as the myotublarins (MTMs). Like Rab7, MTM1 and MTMR2 interact with the WD40 domain of p150 and possibly compete for binding to the same domain. In order to further understand the kinetics of these interactions and obtain information about reaction equilibria and order of assembly of the multicomponent systems, we have developed a novel bead-based flow cytometry assay. We have tested the utility of this assay for monitoring protein-protein interactions using a GST-GFP fusion protein and glutathione loaded flow cytometry suitable sized beads (GSH-beads). The results indicate high-density GSH-beads stably bind GST-GFP only with a very slow off rates that resist several washing steps. Using flow cytometry, we were also able to accurately measure the binding and dissociation constants of GST-GFP to the GSH-beads. Therefore, application of this novel technology to quantitatively monitor the above mentioned interactions between Rab7 and its effector proteins will provide a new mechanistic insights into late endosomal regulation. This technology could also serve as key methodological advance for other protein-protein interaction assays.

1935

**Mapping and Functional Characterization of Phosphorylation Sites on the Endocytic Adaptor Protein Numb**

C. A. Smith,[1] S. Dho,[1] G. Brothers,[2] Y. She,[2] J. McGlade[1]; [1]Labatt Brain Tumor Research Center, Hospital for Sick Children, Toronto, ON, Canada, [2]Advanced Protein Technology Center, Hospital for Sick Children, Toronto, ON, Canada

Regulation of signaling from cell surface receptors is maintained, in part, by tight control over receptor protein levels at the plasma membrane. Receptors can be internalized by constitutive or receptor activation-dependent pathways. Following internalization, receptors are incorporated into intracellular vesicles that results in their removal from the plasma membrane and subsequent down-regulation. The vesicles are then sorted to the lysozome for degradation or recycled back to the cell surface. Attachment of ubiquitin or phosphoryl groups to the activated receptor or associated endocytic proteins often facilitates this sorting process. The adaptor protein Numb plays a role in cell fate determination during development primarily through antagonism of the Notch signaling pathway, and has recently been implicated in both clathrin-dependent and -independent trafficking pathways. The Numb protein contains multiple protein-protein interaction regions including a phosphotyrosine binding (PTB) domain, and precisely conserved endocytic motifs within the carboxy terminus. In *Drosophila*, Numb associates with the serine/threonine kinase NAK (Numb-associated kinase), an enzyme closely related to the adaptor-associated kinase (AAK), a regulator of clathrin mediated endocytosis. This suggests that Numb is a substrate for phosphorylation, and in support of this we observed a mobility shift in the Numb protein following agonist stimulation or with the addition of phosphatase inhibitors, indicating that Numb phosphorylation/dephosphorylation is dynamically regulated. To confirm that Numb is phosphorylated, *in vivo* labeling and phosphopeptide mapping experiments identified five tryptic phosphopeptide fragments in resting cells and two additional fragments following agonist stimulation. Furthermore, mass spectrometry has identified seven individual sites of phosphorylation. Using site-directed mutatagenesis, these residues have been mutated to alanine or glutamic acid to mimic phosphorylation and we are investigating the functional consequences of Numb phosphorylation on stability, localization and receptor trafficking.

1936

**Phosphatases and Kinases Regulate AP2 Binding and Release from Plasma Membrane**

Y. Yim, S. Scarselletta, E. Eisenberg, L. E. Greene; NHLBI, NIH, Bethesda, MD

Three different mechanisms have been described for inducing the dissociation of the clathrin adaptor AP-2 from clathrin-coated pits: phosphorylation of a mu chain tyrosine, dephosphorylation of multiple beta chain serines, and hydrolysis of $PI(4,5)P_2$ on the plasma membrane. To investigate the specific role of each of these mechanisms, we used a cell-free system to which addition of cytosol causes AP2 release and rebinding. While dephosphorylation of AP2 by protein phosphatase 2A (PP2A), which is thought to act on the mu chain, and phosphorylation of the AP2 by casein kinase II which is thought to act on the beta chain, both dissociated AP-2, removal or inhibition of cytosolic PP2A had no effect on AP-2 release. On the other hand, AP2 was dissociated when it was phosphorylated by a cytosolic kinase presumably acting on the beta chain and treatment of the cytosol with ATPgammaS prevented its rebinding suggesting that phosphorylation and dephosporylation is responsible for the AP-2 exchange caused by cytosol. In contrast, in the presence of $Ca^{2+}$ cytosol irreversibly dissociated AP-2. Inhibition of the cytosolic $Ca^{2+}$-activated phosphatase calcineurin prevented this effect and addition of pure calcineurin reproduced it. Calcineruin activates the $PI(4,5)P_2$ phosphatase synaptojanin, which then hydrolyzes $PI(4,5)P_2$. Thus, once $PI(4,5)P_2$ is hydrolyzed, cytosol is no longer able to induce rebinding of AP-2 to the pits. We conclude that $PI(4,5)P_2$ controls the formation and dissolution of clathrin-coated pits while the exchange cycle is controlled by a phosphorylation-dependent release and a dephosphorylation-dependent rebinding of AP2.

1937

**Biophysical Studies of the Yeast Endocytic Scaffold Pan1 to Characterize Phosphorylation-regulated Conformations**

B. Wendland,[1] J. M. McCaffery[2]; [1]Biology, The Johns Hopkins University, Baltimore, MD, [2]Biology and the Integrated Imaging Center, The Johns Hopkins University, Baltimore, MD

The yeast endocytic scaffold protein Pan1 binds to endocytic factors that are required for both early and late endocytic events. We and others have shown that Pan1 binds to clathrin adaptor proteins (early cargo-selection and coat assembly stages) and to actin regulatory factors (late vesicle formation and scission stages). Some of these binding events and the actin polymerization activity are regulated by Prk1-dependent phosphorylation. Pan1 can also interact with itself through a central oligomerization domain to form dimers or higher order oligomers. The N- and C-terminal regions of Pan1 also interact; when the N-terminal region is phosphorylated by the Prk1 kinase, the C-terminal Arp2/3 stimulation activity is blocked. Here, we describe studies that address how Prk1 phosphorylation of Pan1 alters Pan1-Pan1 interactions to yield a range of Pan1 conformations. Electron microscopy of negatively stained purified Pan1 ± Prk1 phosphorylation allowed us to observe distinct morphologies of Pan1 complexes. We also have yeast two-hybrid evidence that Prk1 phosphorylation can have either positive or negative effects on interactions between specific domains of Pan1. Finally, Pan1 purified from *prk1Δ* cells is more prone to proteolysis than is Pan1 purified from wild-type cells, again consistent with the existence of distinct conformations. We are currently assessing Pan1 oligomeric status by equilibrium ultracentrifugation, and are monitoring Pan1 conformations using limited proteolysis and mass spec analyses. A thorough characterization of Pan1 conformations and interactions will help in developing specific models that explain how Pan1 regulates the temporal coordination of proteins mediating the early and

late stages of endocytosis.

1938

**Clathrin Trimer Stability**

S. Perez-Miller, M. W. Drazer, J. A. Ybe; Indiana University, Bloomington, IN

Clathrin is a scaffolding molecule that functions in intracellular trafficking and sorting events. The native clathrin molecule is a pinwheel shaped trimer with rod-like heavy chain 'legs' that interact to form closed baskets around budding vesicles. A clathrin light chain subunit binds along each leg of the trimer, but the function of the clathrin light chain in endocytosis is poorly understood. Additionally, while integrity of the trimer is essential for normal clathrin function, in some cases trimerization has been found to be compromised in the absence of light chains. Our objective is to characterize the role of the clathrin light chain C-terminus. We have used native gel electrophoresis to monitor the oligomerization state of recombinantly expressed bovine clathrin hub (residues 1073-1675) with and without light chains. Clathrin hub alone fails to run as a single species but when the hub is incubated with light chains, the smearing is drastically reduced and a single band is observed. Light chain binding also confers resistance to detergent denaturation of hub. Sequence comparison with a muscle-specific isoform of clathrin that does not bind light chains reveals several amino acid differences in the trimerization domain. We have found that a T1585L substitution in bovine clathrin hub increases trimer stability, as evidenced by a single band on a native gel. We conclude that both light chain binding and specific heavy chain residues are important determinants of clathrin trimer stability. Further, gel filtration chromatography data show that the isolated C-terminal third of light chain binds to the clathrin hub. Based on these data and recently published results from other groups, we have formulated a working model for how the C-terminal third of the light chain contributes to clathrin trimer stability that is currently being tested using biophysical and cell-based approaches.

1939

**Structural Requirements for Signaling Properties of Dynamin-2**

F. Soulet,[1] S. L. Schmid,[1] H. Damke[2]; [1]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Department of Biochemistry, University of Nevada Reno, Reno, NV

The multi-domain GTPase dynamin is essential for endocytic vesicle formation. Increasing experimental evidence supports additional roles for the dynamin GTPases in cellular signaling processes linking dynamin to actin dynamics, cytoskeletal rearrangements, kinase pathways and other signaling events. In mammalian cells dynamin exists in three isoforms with additional splice variants that might attribute to these differential functions and represent forms of specialization. We have previously shown that the ubiquitously expressed isoform dynamin-2 induces p53-dependent apoptosis, when overexpressed ≤5-fold relative to endogenous levels, whereas dynamin-1 does not. Mutants of dynamin-2 that are defective in GTP binding (and defective in endocytosis) do not trigger apoptosis suggesting that increased levels of dynamin-2:GTP rather than protein levels per se are required. Dynamin-2 is 70% identical with the neuronal isoform dynamin-1. With the design of chimeric molecules between dynamin-2 and dynamin-1 we have now addressed the structural and functional requirements responsible for the apoptotic potential of dynamin-2. Surprisingly, not the most divergent C-terminal proline/arginine rich domain (PRD) that interacts with many SH3 domain-containing partners implicated in both endocytosis and signal transduction, but the highly conserved GTPase domain of dynamin-2 is sufficient to trigger apoptosis within the full-length molecule. An assembly-defective mutant of dynamin-2 retains its ability to induce apoptosis suggesting that dynamin-2 can act as a signaling molecule independent on its state of self-assembly. It remains to be shown on which signaling pathways dynamin-2 impinges to ultimately cause apoptosis.

1940

**COPI Subunits Constitute Novel Class E *VPS* Genes Essential for Protein Sorting to Multivesicular Bodies in *Saccharomyces cerevisiae***

G. Gabrielly, R. Kama, J. E. Gerst; Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

Earlier studies proposed a non-conventional role for COPI (coatomer) in endocytosis in mammalian cells. In this study, we examined the requirement for specific COPI subunits in the late steps of endosomal protein sorting in yeast. Initially, we found that carboxypeptidase Y (CPY) was partially missorted to the cell surface in mutants of the COPI B sub-complex (COPIb: Sec27, Sec28, and Sec33), which indicates an impairment in endosomal transport. Next, we observed an accumulation of integral membrane proteins destined for the vacuolar lumen (*i.e.* CPS1, Fur4, and Ste2) at a late endosomal compartment and at the limiting vacuolar membrane in these same mutants. In contrast, Snc1 v-SNARE recycling through early endosomes to the Golgi was unaffected. Labeling with a lipophilic dye, FM4-64, revealed the accumulation of an enlarged endosomal compartment next to the vacuole in mutants of Sec27 and Sec28. These phenotypes are characteristic of class E vacuolar protein sorting (*vps*) mutants, which are impaired in multivesicular body (MVB) protein sorting and biogenesis. Finally, we found a physical interaction between COPIb subunits and MVB-associated protein, Vps27. We propose that COPIb subunit genes are novel members of class E *vps* family and predict their role in protein sorting to MVBs.

1941

**Essential Role of RGS-PX1/Sorting Nexin 13 in Mouse Development and Organization of Endocytic Compartments**

M. G. Farquhar; Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA

RGS-PX1 (also known as sorting nexin 13) is a member of both the regulator of G protein signaling (RGS) and sorting nexin (SNX) protein families. Biochemical and cell culture studies have shown that RGS-PX1/SNX13 is able to attenuate Gαs-mediated signaling through its RGS domain and to regulate endocytic trafficking and degradation of the epidermal growth factor receptor through its sorting nexin activity. To understand the functions of RGS-PX1/SNX13 in vivo, we generated mice carrying targeted mutations of *Snx13* and found that systemic *Snx13*-null mice were embryonic lethal around E12.5. *Snx13*-null embryos had significant overall growth retardation and defects in neural tube closure and blood vessel formation. The mutant placentas were hypoplastic and showed defective labyrinthine layer formation. Moreover, the visceral yolk sac (VYS) endoderm cells in *Snx13*-null embryos showed dramatic changes in the organization of endocytic compartments, presence of autophagic vacuoles and abnormal localization patterns of several endocytic markers, including megalin, an endocytic receptor for multiple nutrients and proteins, ARH, a coat protein that binds clathrin and megalin, LAMP2, a lysosomal protein, and LC3, an autophagy marker. These changes in endocytic compartments in the VYS endoderm suggest that *Snx13*-null embryos are defective in nutrient uptake and transport, which may

contribute to the other developmental abnormalities observed. Taken together, our findings demonstrate an essential role of SNX13/RGS-PX1 in mouse development and provide new insights into its cellular function in the organization of endocytic compartments.

1942

**Retrograde Transport of Bacterial Toxins Through the Recycling Endosome**

J. McKenzie,[1] T. Taguchi,[2] D. Sheff[1]; [1]Pharmacology, Carver College of Medicine, University of Iowa, Iowa City, IA, [2]Osaka School of Medicine, Osaka, Japan

Bacterial toxins from cholera and shigella are heterohexamers that rely on retrograde transport for activity. Each contains a toxic alpha subunit that is delivered to the cytoplasm. Five beta subunits bind cell surface glycolipids and carry the alpha subunit through endosomes, Golgi and ER for cytoplasmic delivery. Golgi entry is rapid, within 45 minutes. Golgi exit is relatively slow, 3-6 hours for complete transfer of shigella toxin B subunits (shiga B). We examined whether Golgi passage is required for ER delivery, or merely kinetically favored thus overshadowing a possible direct endosome to ER pathway. We used $AlCl_4$, an inhibitor of transferrin traffic from the recycling endosome to the basolateral plasma membrane. Surprisingly, treatment also inhibited retrograde traffic of both shiga B and cholera B subunits. Traffic of both into the Golgi was delayed up to 2 hours. Because treatment with fluoride ions disrupts the medial Golgi, this effect may result from Golgi disruption. Therefore, we generated cytoplasts from BSC-1 cells and followed the traffic of shiga B. In cytoplasts lacking Golgi apparatus, the shiga B recycled to the plasma membrane, and was in endosomal structures, but did not colocalize with the ER even after 3 hours. Addition of $AlCl_4$ resulted in strong colocalization of shiga B and transferrin in regenerated recycling endosomes. Together these data suggest that passage through the Golgi is obligatory for both shiga and cholera toxins. Both utilize an $AlCl_4$ sensitive pathway to exit the recycling endosome, and presence of an intact Golgi is not required for exit from the recycling endosomes. Indeed, the sensitivity of both basolateral and retrograde traffic to $AlCl_4$ and re-rerouting of shiga toxin in cytoplasts suggests that the retrograde pathway may share features, such as Rab6 isoform dependence, with the basolateral pathway.

1943

**Syntenin-$PIP_2$ Interaction Controls Arf6-dependent Syndecan Recycling and Cell Spreading**

P. L. Zimmermann,[1] Z. Zhang,[1] G. Degeest,[1] E. Mortier,[1] I. Leenaerts,[1] C. Coomans,[1] J. Schulz,[1] F. N'Kuli,[2] P. Courtoy,[2] G. David[1]; [1]CME, Kuleuven, Leuven, Belgium, [2]Cell Unit, Université Catholique de Louvain, Brussels, Belgium

Syndecans are heparan sulfate proteoglycans that modulate the activity of several growth factors and cell adhesion molecules. PDZ domains in the adaptor protein syntenin interact with the intracellular domain of the syndecans and with the phosphoinositide $PIP_2$, which is involved in the regulation of the actin cytoskeleton and membrane trafficking. Here, we investigated the relevance of the syntenin-$PIP_2$ interaction for the biology of the syndecans. We found that the interaction of syntenin's PDZ domains with $PIP_2$ controls the ADP-ribosylation factor 6 (Arf6)-mediated recycling of syndecan upon cell stimulation. Syntenin defective for $PIP_2$ binding, dominant-negative Arf6 (Arf6 T27N), or dominant-negative phosphatidylinositol 4-phosphate 5-kinase, all block syntenin/syndecan in recycling endosomes. Multiple adhesion and signaling molecules that bind to the heparan sulfate chains of the syndecans are likely to be trapped in these endosomes. FGF receptor accompanies syndecan along the syntenin-mediated recycling pathway, in a heparan sulfate- and FGF-dependent manner. Additionally, we found that syndecans that cannot recycle via the syntenin pathway affect cell spreading. Blocking syntenin-dependent syndecan-recycling using syntenin defective for $PIP_2$ interaction, syntenin loss-of-function, or syndecan defective for syntenin interaction inhibits cell spreading. The effect on spreading depends on the heparan sulphate substitution of the syndecans, suggesting it results from the trapping of syndecan-cargo involved in cell adhesion/signalling rather than from the trapping of syndecan itself. We propose that the link made here between syndecan, heparan sulfate-ligands, syntenin, $PIP_2$ and Arf6-dependent membrane recycling identifies one piece of the molecular framework by which syndecans control cell surface dynamics and behavior.

1944

**A Gene Silencing Strategy to Identify Regulators of Endosomal Cycling**

T. Galvez, J. T. Jones, W. Heo, J. W. Myers, M. Kim, J. Liou, T. Meyer; Molecular Pharmacology, Stanford University, Stanford, CA

Various surface proteins including G protein-coupled receptors, protein tyrosine kinases, channels and transporters cycle between the plasma membrane and endosomal compartments. By regulating the cycling capacities and rates for a specific receptor, cells can control the density of functional receptors at its surface and subsequently the properties of the signal it receives. In order to identify pivotal regulatory players of the membrane recycling machinery, we monitored changes in the properties of this machinery in response to perturbations of the cellular signaling networks using systematic gene silencing. A library of 1920 diced small interfering RNAs (d-siRNAs) was designed to target proteins containing conserved signaling motifs as annotated in the National Center for Biotechnology Information databases. The effect of each silencing was tested on the transferrin receptor (TfR1) recycling pathway in HeLa cells. TfR1 and its ligand cycle in minutes between the cell surface and endosomes and constitute an archetypical model to study endosomal recycling. The perinuclear accumulation after a 10 min pulse of fluorescently-labeled transferrin was measured by automated fluorescence microscopy and image processing and used as the main parameter to detect d-siRNA-induced perturbations. In order to estimate the statistical confidence that a given d-siRNA has a significant effect, we developed a simple variation of ANOVA called *CAsH* (for Confidence Analysis of siRNA Hits) that subtracts the noise distribution of the screen from the actual distribution of the data. Using this parameter we found as expected that multiple hits were known to act in clathrin-mediated endocytosis or in the delivery of receptors to the plasma membrane. Other significant hits are involved in actin polymerization, phosphatidyl-inositol metabolism or PP2A activity. This approach constitutes a framework to investigate how the regulation of endosomal cycling is linked to other cellular functions.

1945

**Arrestins Interact with Multiple ARF-GEFs**

G. Laroche, E. Dupré, J. Parent; Rheumatology Division, University of Sherbrooke, Fleurimont, PQ, Canada

Arrestins have recently been found to mediate a variety of receptor signaling and regulatory processes and to bind to a growing list of signaling and endocytic proteins. Originally characterized as being involved in desensitization of G protein-coupled receptors, arrestins have been shown by numerous studies to have a larger role in the biology of these receptors. Activation of small GTP-binding proteins such as Rho and ARF6 was

shown to be regulated by arrestins. ARF6 and its guanine nucleotide exchange factor (GEF) ARNO are known to bind to arrestins. Here we describe novel interactions between arrestins and multiple GEFs of the ARF1 and ARF6 proteins. The GEFs studied include cytohesin-1, GRP1, EFA6, and ARF-$GEP_{100}$. Co-immunoprecipitation experiments of myc-tagged ARF-GEFs with HA-tagged arrestins carried out in HEK293 cells reveal an interaction between all of the ARF-GEFs studied with arrestin-2 and arrestin-3. However, there is specificity in these interactions. ARF-$GEP_{100}$ interacts equally well with both arrestin-2 and arrestin-3. On the other hand the interaction of cytohesin-1, GRP1, and EFA6 is very strong with arrestin-2 but drastically weaker with arrestin-3. Immunofluorescence microscopy shows that arrestins, GRP1, and cytohesin-1 are distributed uniformly in the cytoplasm. In contrast, EFA6 displays strong plasma membrane localization whereas ARF-$GEP_{100}$ is found both at the membrane and in the cytoplasm. Merging of the images demonstrates that arrestins co-localize in the cytoplasm with GRP1, cytohesin-1 and ARF-GEP100, and weakly with EFA6 in a location that appears just underneath the plasma membrane. Contrary to what we have shown before for ARNO, co-expression of GRP1 and EFA6 do not appear to affect agonist-induced internalization of the G protein-coupled receptor for thromboxane $A_2$ in HEK293 cells. The role of these novel interactions, their specificity, and the possible involvement of arrestins in ARF1 activation will be discussed.

1946

### Gq Directly Interacts with ARNO and Activates ARF6

P. Giguère,[1] M. Rochdi,[1] G. Laroche,[1] E. Dupré,[1] M. R. Whorton,[2] R. K. Sunahara,[2] A. Claing,[3] G. Dupuis,[4] J. Parent[1]; [1]Rheumatology Division, University of Sherbrooke, Fleurimont, PQ, Canada, [2]Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI, [3]Department of Pharmacology, University of Montreal, Montreal, PQ, Canada, [4]Graduate Program of Immunology, University of Sherbrooke, Fleurimont, PQ, Canada

G-protein coupled receptors are widely expressed hepta-helical receptors with pleiotropic effects which are tightly regulated. Here we report a novel aspect of their regulation by a direct molecular interaction between a heterotrimeric G protein and a guanine nucleotide exchange factor (GEF) for the small GTPases of the ADP-ribosylation factor family (ARFs), namely ARNO (ARF nucleotide binding site opener). Immunofluorescence microscopy in HEK293 cells showed that Gq and ARNO co-localized intracellularly in the basal state. On the other hand, the constitutively active Gq-R183C mutant localized to the plasma membrane and induced the translocation of ARNO from intracellular compartments to the plasma membrane. Interestingly, co-immunoprecipitation studies revealed that ARNO and ARF6 formed complexes preferentially with activated Gq compared to non-activated Gq. Binding experiments using purified proteins showed that Gq interacted directly with ARNO. Agonist treatment of the Gq-coupled thromboxane $A_2$ receptor triggered ARF6 activation, which was prevented by the sequestration of Gq. We previously demonstrated that internalization of this receptor required Gq signaling through a phospholipase Cβ- and protein kinase C-independent mechanism. Here we show that expression of ARNO and ARF6 promoted, whereas dominant negative mutants of these proteins inhibited the internalization of the thromboxane $A_2$ receptor. Taken together, our results indicate that stimulation of the thromboxane A2 receptor activates Gq which in turn leads to activation of ARF6 through ARNO, ultimately triggering internalization of the receptor. Thus in the present study, we elucidate for the first time how a heterotrimeric G protein can activate a small G protein of the ARF family. This mechanism could be a widely used signaling pathway involved in the regulation of intracellular trafficking events.

1947

### Mutational Analysis of *Drosophila* Rab GDI and its Interaction with Gint3, a UBX-domain Containing Protein

D. J. Holtzman, A. K. Moore, A. Chen, B. E. Richardson, K. L. Ayres, N. F. Sangji, M. L. Keese, C. M. Cheney; Biology, Pomona College, Claremont, CA

Rab GTPases play multiple roles in vesicle transport. Rab GDP dissociation inhibitor (GDI) regulates rab placement by retrieving rabs from target membranes and delivering rabs through the cytoplasm to donor membranes. GDI plays a crucial role in ensuring that the correct rab is placed in the correct membrane. It is hypothesized that the GDI-rab complex must interact with a membrane bound receptor complex that triggers the release of rab from GDI. To identify proteins in such a complex, we have carried out screens for GDI interactors, using Drosophila as the model system, since the fly genome has a single GDI, but multiple rabs. In a yeast two-hybrid (Y2H) screen, using wild-type GDI in a pLexA vector as bait and screening a Drosophila cDNA library, a protein with a ubiquitin-like UBX domain was identified as a GDI interactor. This protein was named Gint3, for GDI interacting protein 3. Gint3 has a PUG N-glycanase-associated domain and a UBX ubiquitin-like domain, but no obvious transmembrane domain. Gint3 interacted with L319 mutant GDI, which has a mutation that alters the lipid-binding pocket of Domain II of GDI (Ricard et al, Genesis 31:17), but did not interact with a non-specific prey, human lamin C. Interaction was also seen when Gint3 was placed in the bait vector and GDI in the prey vector. GDI interacted with Gint3 in a LexA Y2H system as well as in a GAL4 Y2H system. Phylogenetic analysis of Gint3 and UBX domain proteins shows that Gint3 forms a distinct subfamily of UBX domain proteins with the highest bootstrap value. Supported by NSF-RUI grant 0212730 to CMC.

1948

### RhoB Effector Mutants and their Effects on Post-Endocytic Traffic in Polarized MDCK Cells

C. Rondanino, W. G. Ruiz, C. J. Mazur, G. Apodaca; Medicine/ Cell Biology and Molecular Physiology, University of Pittsburgh, Pittsburgh, PA

Endocytic traffic is highly regulated in epithelial cells. RhoB, a member of the Rho family of GTPases, modulates post-endocytic events in many cells including polarized Madin-Darby Canine Kidney (MDCK) cells. In those cells, expression of a dominant active mutant of RhoB (RhoBV14) decreases transcytosis, receptor recycling and degradation while a dominant negative mutant of RhoB (RhoBN19) has a stimulatory effect. However, the mechanism by which RhoB mediates these processes is unknown. We hypothesize that RhoB acts through downstream effectors. We have stably expressed RhoBV14 bearing point mutations in the effector binding domain. Such mutations selectively disrupt RhoB binding to various known effectors: Rho-kinase, Rhophilin, Kinectin, Citron, mDia, and PKN. The effect of these mutants on post-endocytic traffic was measured. In cells overexpressing RhoBV14Y37 (which cannot bind any known RhoB effectors), we observed a significant slowing of IgA transcytosis and a decrease in the plateau level by 15% relative to control non-expressing cells. Transferrin recycling was also slowed but the absolute amount of recycled transferrin was not altered. These results suggest that unknown RhoB effectors are involved in the regulation of those pathways. Similar results were obtained with the mutant RhoBV14A39 that interacts specifically with mDia, suggesting that this protein has no regulatory role. Interestingly, in cells that express RhoBV14V39 that binds to both mDia and Rho-kinase, the extent of IgA transcytosis and

transferrin recycling was impaired by approximately 30% and 15%, respectively. We propose that RhoB acts through yet unknown effectors to modulate post-endocytic traffic in MDCK cells and that Rho-kinase could also be involved as well. We are currently conducting a yeast-two hybrid experiment to identify novel RhoB binding partners.

1949

### The *Tetrahymena thermophila* Phagosome Proteome

L. A. Klobutcher,[1] M. E. Jacobs,[1] L. DeSouza,[2] K. W. M. Siu[2]; [1]MMS Biology, University of Connecticut Health Center, Farmington, CT, [2]Department of Chemistry and Centre for Research in Mass Spectrometry, York University, Toronto, ON, Canada

In vertebrates, phagocytosis plays a role in the immune response, tissue development, and the removal of apoptotic cells. In lower eukaryotes, phagocytosis provides a means of ingesting other microorganisms to meet the nutritional needs of the cell. Research in a number of experimental systems indicates that phagocytosis is a multistep process that involves hundreds of genes and proteins, but the identities and functions of the proteins mediating phagocytosis are still incompletely understood. As a result, we have been analyzing the protein composition of phagosomes in the ciliate *Tetrahymena*. To purify phagosomes, the *Tetrahymena* cells were first fed latex beads. Following lysis of the cells, sucrose gradient centrifugation was employed to separate the bead-filled phagosomes from other cellular components. Proteins and/or tryptic peptides derived from the phagosomal preparations were then subjected to three different two-stage fractionation procedures followed by tandem mass spectrometry (MS/MS). The resulting mass spectra were compared to either conceptual translations of the entire *Tetrahymena* macronuclear genome sequence or the predicted total cellular proteome to identify proteins present in phagosomes. To date, more than 80 proteins with significant similarity to proteins of known function have been identified. This includes more than 25 proteins known to be associated with phagosomes in other experimental systems, providing an indication that *Tetrahymena* phagocytosis shares similarities with other organisms. In addition, 17 proteins proteins of unknown function have been identified, which are candidates for novel phagosome proteins. We are now beginning to utilize the strong genetic/molecular genetic tools available in *Tetrahymena* to generate strains with knockout or knockdown mutations in putative phagosome genes to obtain insight into their functions. In addition, strains with GFP-tagged versions of phagosome proteins are being generated to determine their cellular localization.

## Protein Targeting to the Endocytic Pathway (1950-1967)

1950

### Effector Binding Determines Rab GTPases Localization

D. A. Aivazian, R. L. Serrano, S. R. Pfeffer; Biochemistry, Stanford University, Stanford, CA

Rab GTPases regulate transport vesicle formation, motility, docking and fusion. Hypervariable domains are thought to specify the distinct localizations of Rab GTPases. We have found that the primary determinant of Rab localization is effector interactions, which need not involve hypervariable domain sequences. Effectors are proteins that bind preferentially to GTP-bearing Rabs. Rab9, Rab5 and Rab1 chimerae, containing heterologous hypervariable domains were generated; we quantitated their ability to bind a number of Rab effectors. In all cases, intracellular localization of the chimerae correlated with the capacity for specific Rab effector interaction. Moreover, intracellular concentrations of Rab effectors determined the localization of two different chimeric Rabs that could bind to the effectors of two different Rab proteins. These data rule out a simple model for an organelle-specific Rab receptor, and support the view that Rabs participate in complex and dynamic, protein:protein and protein:lipid, microdomain interactions that are important both for steady state Rab localization and the specialized functions of subcellular compartments.

1951

### Involvement of Rab5 and Rab11 in the Trafficking of H-Ras to Recycling Endosomes in CHO-K1 Cells

G. A. Gomez, J. L. Daniotti; Departamento de Química Biológica, CIQUIBIC (CONICET-UNC), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

H-, N- and K-Ras are isoforms of Ras proteins, which undergo different lipid modifications at the C-terminus. These post-translational events make possible the association of Ras proteins both with the inner plasma membrane and to the cytosolic surface of endoplasmic reticulum and Golgi complex, which is also required for the proper function of these proteins. To better characterize the intracellular distribution and sorting of Ras proteins, constructs were engineered to express the C-terminal domain of H- and K-Ras fused to variants of green fluorescent protein. Using confocal microscopy, we found in CHO-K1 cells that H-Ras, which is palmitoylated and farnesylated, localized at the recycling endosome in addition to the inner leaflet of the plasma membrane. In contrast, K-Ras, which is farnesylated and non-palmitoylated, mainly localized at the plasma membrane. We demonstrate that sorting signals of H- and K-Ras are contained within the C-terminal domain of these proteins, and that palmitoylation on this region of H-Ras might operates as a dominant sorting signal for proper subcellular localization of this protein. Using selective photobleaching techniques, we demonstrate the dynamic nature of H-Ras trafficking to the recycling endosome from plasma membrane. We also provide evidence that Rab5 and Rab11 activities are required for proper delivery of H-Ras to the endocytic recycling compartment. Using a chimera containing the Ras binding domain of c-Raf-1 fused to a fluorescent protein, we found that a pool of GTP-bounded H-Ras localized on membranes from Rab11-positive recycling endosome after serum stimulation. These results suggest that H-Ras present in membranes of the recycling endosome might be activating signal cascades essential for the dynamics and function of the organelle. Supported by: Fundación Antorchas, FONCYT, CONICET, SECyT-UNC

1952

### The Dileucine Motif and AP-2 are Required for Efficient Endocytosis of $\beta_2$-adrenergic Receptors

H. O. Awwad, B. J. Knoll; Pharmacological & Pharmaceutical Sciences, University of Houston, Houston, TX

Agonist-activated $\beta_2$-adrenergic receptors ($\beta_2$ARs) recruit β-arrestin, a scaffolding protein, to form a β-arrestin-receptor complex. This complex then recruits adaptor proteins, mainly AP-2, at the plasma membrane for clathrin-mediated endocytosis. AP-2 is a heterotetrameric adaptor protein complex that binds to β-arrestin, clathrin, phosphoinositides and certain transmembrane proteins. The dileucine sorting motif is a recognition signal

for AP-2 binding. The fourth intracellular loop of $\beta_2$ARs has a conserved dileucine motif, at residues 339 and 340, known to be involved in receptor internalization. The role of AP-2 has been studied using dominant negative mutational analyses. However, the effect of silencing AP-2 by RNA interference on $\beta_2$AR internalization has not been studied yet. Our objective was to study the effect of AP-2 silencing on the internalization of $\beta_2$ARs and mutant $\beta_2$ARs with the dileucines changed to dialanines (LLAA). HEK293 cells stably expressing $\beta_2$ARs or LLAA $\beta_2$ARs were treated with short interfering RNA (siRNA) targeting the μ2 subunit of AP-2. By immunofluorescence microscopy, we show that silencing AP-2 inhibits agonist-induced $\beta_2$AR internalization, as well as transferrin internalization. LLAA $\beta_2$ARs showed reduced internalization in radioligand binding assays, which was further reduced upon treatment with siRNA targeting the AP-2. In addition, LLAA $\beta_2$ARs show reduced G-protein coupled receptor kinase (GRK) site phosphorylation and slower β-arrestin recruitment. The absence of either the dileucine motif or AP-2 complex alone reduces $\beta_2$AR internalization, however the absence of both almost abolishes $\beta_2$AR internalization in an additive manner. We conclude that both the dileucine motif and AP-2 complex are required for efficient $\beta_2$AR internalization. Supported by a grant from the American Heart Association, Texas Affiliate 0455072Y

1953

**Identification of Sorting Determinants at the C-Terminal Cytosolic Tail of Mucolipin-1**

S. Vergarajauregui, R. Puertollano; Laboratory of Cell Signaling, NHLBI, National Institutes of Health, Bethesda, MD

Mucolipidosis type IV (MLIV) is an autosomal recessive lysosome storage disorder characterized by severe psychomotorretardation and oftalmological abnormalities, including corneal opacity, retinal degeneration, and strabismus. Unlike the situation in other lysosomal disorders, the accumulation of heterogeneous storage material observed in MLIV does not result from the block in the catabolic pathways, but is due to a transport defect in the late steps of endocytosis. MCOLN1, the gene mutated in MLIV patients, encodes a protein called mucolipin-1 that functions as a Ca2+ permeable channel and has been implicated in the biogenesis of lysosomes. However, it is important to note that very little is known about the factors that regulate the intracellular trafficking and final distribution of mucolipin-1. In this study we have analyzed the presence of sorting motifs in the C-terminal tail of mucolipin-1 by using a chimera consisting of the extracellular and transmembrane regions of Tac fused to the C-terminal cytosolic tail of mucolipin-1. We have found that the C-terminal tail of mucolipin-1 contains sorting information for location at early endosomes. This localization is dependent on a di-leucine motif, which mediates internalization of the Tac-MLN Tail chimera. Mutation of the di-leucine to alanines or depletion of AP2 from Hela cells caused retention of Tac-MLN Tail at the plasma membrane. Also, the C-terminal tail of mucolipin-1 fused to GFP associates with intracellular membranes. It seems that palmitoylation of three cysteine residues mediates recruitment of GFP-MLN Tail to membranes. Mutation of these cysteines to alanines inhibits internalization of Tac-MLN Tail. We concluded that the C-terminal tail of mucolipin-1 contains a di-leucine internalization motif that mediates the endocytosis of Tac-MLN Tail chimera through an AP2 dependent pathway and palmitoylation might play a role on mucolipin-1 trafficking by promoting endocytosis.

1954

**The FDNPVY Sequence of LDLR Is Not Required for VLDL Metabolism in Fibroblasts**

P. Michaely,[1] W. Li,[1] R. Anderson,[1] H. Hobbs,[2] J. Cohen[3]; [1]Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX, [2]Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX, [3]Human Nutrition, University of Texas Southwestern Medical Center, Dallas, TX

The LDLR is the principal receptor that mediates the clearance of LDL and VLDL from the circulation. Internalization of LDL requires the $FDNPVY^{807}$ sequence on the LDLR cytoplasmic tail and mutation of the tyrosine to cysteine (Y807C) is associated with hypercholesterolemia. Here, we tested whether the FDNPVY sequence is also required for VLDL binding, uptake and degradation. Fibroblasts from individuals expressing either normal, Y807C or no LDLR ($LDLR^{-/-}$) were studied. Using $^{125}$I-VLDL assays, we show that the Y807C mutation had little effect on the ability of fibroblasts to bind or degrade VLDL. Uptake was examined by electron microscopy using colloidal gold labeled VLDL (VLDL-gold) and we observed that VLDL-gold accumulated in late endosomes of Y807C cells in a manner similar to that of normal cells. This internalization was specific to VLDL because LDL-gold did not accumulate in late endosomes of Y807C fibroblasts and $^{125}$I-LDL degradation was sharply reduced in these cells. Uptake and degradation of VLDL was LDLR dependent because we did not observe uptake or degradation of $^{125}$I-VLDL or VLDL-gold in $LDLR^{-/-}$ cells. We next examined the surface distribution of LDLR and the distribution of LDLR bound LDL and VLDL using LDLR immunogold, LDL-gold and VLDL-gold, respectively. We observed similar enrichment of VLDL in coated pits of both normal and Y807C cells, while only coated pits of normal cells were enriched with LDL and LDLR. We conclude that the FDNPVY sequence of the LDLR is not required for VLDL to be targeted to coated pits, internalized and degraded.

1955

**Biosynthetic and Endocytic Targeting to the Recycling Endosomal Compartment Involves a Critical Glutamic Acid Residue within the Cytoplasmic Tail of Prostate Specific Membrane Antigen**

J. J. Christiansen,[1] J. R. Goldenring,[2] A. K. Rajasekaran[1]; [1]Department of Pathology and Laboratory Medicine, University of California, Los Angeles, Los Angeles, CA, [2]Department of Surgery, Vanderbilt University, Nashville, TN

Prostate specific membrane antigen (PSMA) is a 100kDa glycoprotein with a highly restricted profile of tissue expression. In addition to the benign prostatic epithelium, PSMA is expressed in tumor-associated neovasculature and at increased levels in most cases of prostate cancer. We have previously demonstrated PSMA is targeted for clathrin-mediated endocytosis via a novel MXXXL motif and subsequently recycled back to the cell surface. The recycling of membrane proteins and receptors may either occur directly from the early endosomes or via a more indirect route. Some membrane proteins are first targeted to a tubulovesicular membrane compartment, referred to as the recycling endosomal compartment, or REC. The REC is a microtubule dependent structure proximal to the nucleus and associated with the centrosomes in both polarized and nonpolarized epithelial cells. The REC contains proteins internalized from both the apical and basolateral plasma membrane surfaces of polarized epithelial cells, however, the mechanisms and signals responsible for targeting of proteins to the REC is not well understood. In the current study, we demonstrate that PSMA is localized to the REC and that targeting to this compartment requires a critical glutamic acid residue within the cytoplasmic domain. Furthermore, our data indicate that while mutation of the PSMA endocytic motif effectively abolishes internalization from the plasma membrane, these mutant forms of PSMA continue to localize to the REC at steady state. Thus, we provide evidence that targeting to the REC can occur

independent of endocytosis and provide insight into the regulation and dynamic nature of this intermediate trafficking compartment.

1956

**The Endosome-Associated Protein Hrs is Hexameric and Exists to Increase Cargo Trafficking**

L. Pullan,[1] S. Mullapudi,[1] Z. Huang,[1] P. R. Baldwin,[1] C. Chin,[2] W. Sun,[3] S. Tsujimoto,[3] S. J. Kolodziej,[4] J. K. Stoops,[4] J. C. Lee,[2] M. N. Waxham,[3] A. J. Bean,[3] P. A. Penczek[1]; [1]Biochemistry and Molecular Biology, University of Texas - Houston Medical School, Houston, TX, [2]Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX, [3]Neurobiology and Anatomy, University of Texas - Houston Medical School, Houston, TX, [4]Pathology, University of Texas - Houston Medical School, Houston, TX

The first structure of the endosomal associated protein, Hrs, has been determined using cryo-electron microscopy and single particle analysis. Hrs physically interacts with a number of proteins, including eps15, SNX-1, and SNAP-25 that are involved in membrane trafficking. It also interacts with STAM1 forming a complex which binds ubiquitin moieties and acts as sorting machinery that recognizes ubiquitinated receptors and transfers them to further sequential lysosomal sorting/trafficking processes. Through the interaction with SNAP-25, Hrs is able to inhibit endosomal fusion. Analytical ultracentrifugation has revealed that Hrs exists as a hexamer in cells with no evidence for the monomeric state. The purified hexameric form of the Hrs was imaged using a 300 kV electron microscope and its 3-D structure was determined using single particle reconstruction method. First, using principal component analysis of the two-dimensional EM images we have determined that the symmetry of the hexameric structure is D3. Subsequently, we determined the three-dimensional EM density map that revealed that the structure of the hexameric Hrs is barrel-shaped with a central core and distinct caps on either end of the barrel, and is made up of three antiparallel dimers. With a slight rearrangement of its quaternary structure, the crystal structure of the FYVE and VHS domains fits into the Hrs end caps in the EM density map. This clearly indicates that the domains which interact with the endosomal membrane are located to facilitate the anchorage of Hrs to the membrane, thereby, initiating the functional processes of Hrs on the endosome. Based on our model, the location of the individual UIM domains of Hrs within the hexamer allows for increasing levels of ubiquitin binding and therefore allows for greater trafficking of cargo via endocytosis.

1957

**Phosphatidic Acid Induces Endocytosis of Inactive EGF Receptors through the cAMP/PKA Pathway**

A. Silva, A. Norambuena, J. Valenzuela, A. González; Depto. Reumatología. Fac. Medicina. Centro de Regulación Celular y Patología, Fac. Ciencias Biologicas. P. Universidad Católica de Chile and MIFAB., Santiago, Chile

The endocytic pathway provides mechanisms for attenuating external stimuli as well as bestowing temporal and spatial determinants on the EGFR signaling system. Ligand-binding activates the intrinsic tyrosine-kinase activity of the receptor and induces endocytosis of active receptors. We have described that inhibition of basal PKA activity induces endocytosis of EGFR in the absence of ligand. Therefore, the endocytic pathway could also serve to control the permanency of empty EGFR at the cell surface through a mechanism sensitive to the activation status of the cAMP/PKA system. Phospholipase D (PLD) through its product phosphatidic acid (PA) participates in several vesicular trafficking processes, including the ligand-induced EGFR endocytosis, via activation of specific phosphatidylinositol kinases. However, PA also activates the phosphodiesterase (PDE) 4D3, from the PDE4 family of rolipram-sensitive PDEs that degrade cAMP. We found that: a) propranolol and disipramine used as inhibitors of the enzyme that degrades PA, provoked the expected increase in PA levels and decrease in cAMP levels and PKA activity, while increasing the endocytosis of inactive EGFR; b) Micelles of PA also induced EGFR endocytosis; c) The constitutive endocytosis of transferrin and the ligand-induced endocytosis of mu-opioid receptors did not respond to PA; c) forskolin and rolipram diminished the EGFR endocytosis induced not only by propranolol but, strikingly, also by EGF. These results indicate that a PDE4-mediated link between the PA and the cAMP/PKA systems constitutes a novel EGFR regulatory mechanism, that can reversibly control the relative distribution of empty and inactive EGFR between the cell surface and endocytic compartments. This mechanism is potentially amenable to modulation by the multiple factors that can increase the cellular PA levels, including stimuli to the EGFR itself (Financed by FONDAP grant: 13980001 and MIFAB).

1958

**Regulation of EGF Receptor Downregulation by UBPY-mediated Deubiquitination at Endosomes**

E. Mizuno, T. Iura, A. Mukai, N. Kitamura, M. Komada; Department of Biological Sciences, Tokyo Institute of Technology, Yokohama, Japan

Ligand-activated receptor tyrosine kinases undergo endocytosis and are transported to endosomes, where they are sorted for lysosomal degradation. This “receptor downregulation” process is crucial to terminate the cell proliferation signals produced by activated receptors. At endosomes, ubiquitination of the receptors serves as a lysosomal sorting signal, which is recognized by a complex of two ubiquitin-binding proteins, Hrs and STAM. We have previously shown that the Hrs-STAM complex binds a functionally uncharacterized deubiquitinating enzyme UBPY (ubiquitin-specific protease Y)/USP8 (ubiquitin-specific protease 8). In this study, we examined the role of UBPY in the downregulation of epidermal growth factor receptor (EGFR). UBPY overexpression reduced the ubiquitination level of EGFR and delayed its degradation in EGF-treated cells. Immunopurified UBPY also deubiquitinated EGFR in vitro. UBPY bound to ubiquitinated EGFR in EGF-treated cells, whereas a UBPY mutant that cannot associate with the Hrs-STAM complex did not. In addition, the binding of UBPY to EGFR was reduced in Hrs-depleted cells, suggesting that the interaction with the Hrs-STAM complex is required for UBPY to interact with EGFR. Immunostaining of HeLa cells using anti-UBPY antibody showed that endogenous UBPY localized on exaggerated endosomes caused by the overexpression of Hrs or a dominant-negative mutant of SKD1, proteins that play roles in the endosomal sorting of ubiquitinated receptors. In addition, a catalytically-inactive UBPY mutant clearly localized on endosomes in normal as well as in Hrs-depleted cells, suggesting that the endosomal localization of UBPY is not mediated by the Hrs-STAM complex. Finally, the ubiquitination level of ligand-activated EGFR was elevated and its degradation was accelerated in cells in which endogenous UBPY was depleted by RNAi. These results suggest that UBPY deubiquitinates EGFR on endosomes and regulates the rate of EGFR downregulation.

1959

***FcRn* as a Regulator of *IgG* Trafficking: Analyses of Intracellular Sorting Events**

P. Prabhat*,[1,2] Q. Tang*,[1] Z. Gan,[1] Y. Chao,[1] R. J. Ober,[1,2] E. S. Ward[1]; [1]Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX, [2]Department of Electrical Engineering, University of Texas at Dallas, Richardson, TX

The Fc receptor, FcRn, plays a pivotal role in regulating the serum levels of immunoglobulin G (IgG) in mice, and much data support the concept that the human ortholog serves a similar function. FcRn also transports IgGs across cellular barriers to diverse body sites. The current model for FcRn function is that following uptake of IgGs into cells by fluid phase pinocytosis, IgGs bind to FcRn in early endosomes and are salvaged from lysosomal degradation (1). FcRn bound IgGs are subsequently trafficked to the cell surface and released by exocytosis (2). The question arises as to how FcRn acts as a salvage receptor in cells. The pH dependence of the IgG-FcRn interaction (tight binding at pH 6.0 that becomes progressively weaker as near neutral pH is approached) distinguishes this interaction from other classically studied receptor-ligand systems for which the trafficking pathways have been elucidated. In the current study we have used biotinylated IgG-quantum dot complexes in combination with live cell fluorescence imaging of FcRn-GFP transfected endothelial cells to analyze the sorting events in early endosomes. The use of quantum dots has allowed us to track intracellular processes for relatively long time periods, which in turn has led to insight in the molecular mechanisms of endosomal sorting. 1. Ober, R.J. *et al., J. Immunol.,* **172**, 2021-2029 (2004). 2. Ober, R.J. *et al., Proc. Natl. Acad. Sci., USA*, **101**, 11076-11081 (2004). * These authors contributed equally. Supported by grants from the NIH (R01 AI39167, R01 AI50747 and R01 AI55556).

1960

**Analyses of the Involvement of the *Fc* Receptor, *FcRn*, in the Trafficking of Albumin in Human Endothelial Cells**

Q. Tang,[1] Z. Gan,[1] J. Zhou,[1] C. Vaccaro,[1] R. J. Ober,[1,2] E. S. Ward[1]; [1]Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX, [2]Department of Electrical Engineering, University of Texas at Dallas, Richardson, TX

Albumin is the most abundant serum protein that serves to regulate both the osmotic balance and transport a wide array of proteins, lipids and metabolites to different body sites. Despite the important functions of albumin, the mechanisms that maintain relatively constant serum levels of this protein are poorly understood. Recent studies in mice have suggested that the MHC Class I-related receptor, FcRn, plays a role in controlling albumin levels in an analogous way to that described for immunoglobulin G (IgG) (1). For IgG, FcRn has been shown to act as a salvage receptor that binds to pinocytosed IgG in early endosomes and salvages the bound ligand from lysosomal degradation (2). FcRn bound IgGs are subsequently trafficked to the cell surface and released by exocytosis (3). In the current study we have used live cell fluorescence imaging of FcRn-GFP transfected endothelial cells to analyze the intracellular trafficking of Alexa (546 or 647) labeled albumin. Triple color experiments have also been used to compare the intracellular routes taken by albumin with those followed by IgGs of different affinities for FcRn binding. Our studies provide insight into the processes that are involved in albumin trafficking. 1. Chaudhary, C. *et al., J. Exp. Med.,* **197**, 315-322 (2003). 2. Ober, R.J. *et al., J. Immunol.,* **172**, 2021-2029 (2004). 3. Ober, R.J. *et al., Proc. Natl. Acad. Sci. USA*, **101**, 11076-11081 (2004). Supported by grants from the NIH (R01 AI39167, R01 AI50747 and R01 AI55556).

1961

**The Exocyst Complex Regulates Multiple Post-Endocytic Trafficking Pathways in Polarized MDCK Cells**

A. Oztan,[1] S. Hsu,[2] C. Yeaman,[3] G. Apodaca[1]; [1]Department of Medicine, University of Pittsburgh, Pittsburgh, PA, [2]Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ, [3]Department of Anatomy and Cell Biology, University of Iowa, Iowa City, IA

The exocyst is an octomeric complex that regulates delivery of newly synthesized proteins to the basolateral plasma membrane domain of polarized Madin-Darby canine kidney (MDCK) cells. In these cells, the exocyst has been variously localized to the lateral plasma membrane near the junctional complex, the trans-Golgi network, and transferrin-positive recycling endosomes. At present, it is unknown whether the exocyst is localized to additional endocytic compartments or whether it regulates transport within the endosomal system of polarized epithelial cells. Analysis of the distribution of the exocyst subunits Sec6, Sec8, and Exo70 in polarized MDCK cells by confocal microscopy confirmed that in addition to the lateral membrane, these proteins were partially localized to vesicles that are positive for the basolateral recycling marker transferrin. There was also a significant subapical pool of Sec6/Sec8/Exo70 that showed extensive colocalization with transcytosing IgA and the apical recycling endosome marker Rab11. Preliminary immunoisolation experiments confirm that Sec8 is found on Rab11-positive endosomes. Streptolysin-O (SLO)-permeabilized MDCK cells were used to analyze the involvement of the exocyst complex in endocytic trafficking pathways. Recycling of transferrin in this assay was inhibited 57.5 ± 4.0% by the addition of function blocking Sec8 antibodies, but was not effected by the addition of a non-specific control antibody. Furthermore, Sec8 antibodies inhibited IgA transcytosis 42.6 ± 1.9% and IgA recycling 27.5 ± 3.0%. In addition to the previously described role for the exocyst complex in regulating basolateral delivery of biosynthetic cargo, these results indicate that the exocyst complex of polarized MDCK cells is localized to endocytic recycling compartments where it regulates basolateral recycling, basolateral-to-apical transcytosis and apical recycling pathways.

1962

**Sorting Nexin 5 and Retromer Recruitment to the Maturing Macropinosome**

M. C. Kerr,[1] R. Leutterforst,[1] N. Hamilton,[1] M. Lindsay,[1] F. Simpson,[1] R. G. Parton,[1] P. A. Gleeson,[2] R. D. Teasdale[1]; [1]Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia, [2]Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, Australia

Stimulation of cells with epidermal growth factor induces a change in the subcellular distribution of sorting nexin 5. Using a combination of real-time and immuno-fluorescence microscopy we have characterised the subsequent association of SNX5 with enlarged endocytic macropinosomes and tracked the progression of this endocytic compartment through its early stages of maturation using SNX5 as a marker. Initially SNX5 is recruited to distinct subdomains of the compartment as intense patches on the cytoplasmic face of the macropinosome. These patches then traverse the cytoplasmic face of the compartment in a directed manner before being incorporated into highly dynamic, often branched, tubular structures. Extension of the tubular subcompartment is dependent on the microtubule network. Concurrent with the extension and departure of the tubular elements is a marked reduction in the surface area and volume of the macropinosome. Characterisation of this compartment revealed its role in the early endocytic trafficking pathway from the plasma membrane to the early endosome. We demonstrate that the macropinocytic pathway characterised is epidermal growth factor receptor and transferrin receptor positive and is a novel site of membrane recruitment for the human retromer complex subunits; Vps26A, Vps29 and SNX1. Furthermore, we find that SNX5 interacts with the retromer complex via the formation of heteroligomers with SNX1 and cannot form homoligomers. This association represents the first observation of the retromer's involvement in macropinocytosis and provides a model for the characterisation of early-stage endosome maturation in real-time.

1963

**Membrane-associated Trafficking of Stat3 in the Cytoplasm: The Stat3 Signaling Endosome Hypothesis**

M. Shah, P. B. Sehgal; Cell Biology and Anatomy, New York Medical College, Valhalla, NY

Cytokine- and growth factor-initiated STAT signal transduction from the plasma membrane to the nucleus is a vital event in numerous cellular processes. Evidence from this lab and others has shown that IL-6/STAT3 signaling is initiated at the level of plasma membrane raft microdomains, but the cytoplasmic itinerary remains unclear. STAT signaling is widely represented as a free cytosolic process. We re-evaluated the question of membrane-associated trafficking of STAT3 in the cytoplasm. Cell fractionation, immunofluorescence and biochemical data showed constitutive as well as IL-6-enhanced association of cytoplasmic STAT3 as well as PY-STAT3 with vesicular components ("signaling endosome") which partially overlapped with markers of the caveolar/endolysosomal pathways [clathrin heavy chain (CHC), caveolin-1, Rab5, Rab7, LAMP1, LAMP2 and EEA1] in hepatocyte, endothelial, epithelial and smooth muscle cells. The endocytosis inhibitor phenylarsine oxide induced a dramatic increase in bulk STAT3 association with cytoplasmic membranes and between STAT3 and CHC. Additionally, the previously reported cytosolic 1-2 MDa STAT3 ("statosome II") complexes contained CHC, suggesting a clathrin-like cycling of STAT3 between cytosol and membrane elements. We employed a detergent dissection approach to determine the relative magnitude of membrane-associated versus free cytosolic PY-STAT3 signaling. Using this approach, approximately 70 % of IL-6-activated cytoplasmic PY-STAT3 was found to be associated with cytoplasmic vesicular elements ("STAT3 signaling endosomes), suggesting that the membrane-associated signaling is the major pathway. Inhibitors and expression constructs of proteins affecting raft/endolysosomal trafficking provided data indicating that this pathway included both a transcriptional activation component and a signal inhibitory component. These observations require a modification of our understanding of the cytoplasmic physiology of STAT3 to include vesicular trafficking.

1964

**A Lethal Mutation in the NEUZ/NHR Domain of Neuralized Alters Protein Subcellular Localization and the Ability to Colocalize with the Notch Ligand, Delta**

C. Commisso,[1,2] G. L. Boulianne[1,2]; [1]Developmental Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]Medical and Molecular Genetics, University of Toronto, Toronto, ON, Canada

The Notch signaling pathway is involved in numerous developmental cell fate decisions and when signaling is disrupted a neurogenic phenotype results as a consequence of excess neural determination. The core pathway consists of the Notch receptor and its ligands, Delta and Serrate/Jagged. Neuralized is an E3 ubiquitin ligase thought to non-autonomously activate the Notch signal by targeting the Notch ligands for ubiquitin-mediated endocytosis. Neuralized is expressed as two isoforms, each containing a C-terminal RING domain and an internal repeat of the NEUZ/NHR domain, a conserved protein domain found in over 80 Neuralized-related proteins within the animal kingdom. We have previously shown that Neuralized functions as an E3 ubiquitin ligase *in vitro*, and that this function is dependent upon an intact RING domain. Here we show that a lethal, neurogenic allele of *neuralized* is a result of a mutation in a conserved residue of the NEUZ/NHR domain, affecting both of the Neuralized isoforms. The resulting mutant proteins exhibit altered subcellular localization in *Drosophila* S2 cells and *in vivo*. Other groups have shown that Delta and Neuralized are found in a complex and we have determined that Neuralized proteins normally colocalize with a wild-type or a membrane-tethered form of Delta in S2 cells. Here we show that the NEUZ/NHR mutants are defective in Delta colocalization. Taken together, this data suggests that the NEUZ/NHR domains may be facilitating protein-protein interactions that are important to subcellular localization and ubiquitin ligase target recognition. This is the first evidence of a possible function for the NEUZ/NHR protein domain and our current efforts include elucidating the specific protein-protein interactions that have been abrogated in NEUZ/NHR mutants, causing them to be mislocalized.

1965

**Localized PtdIns 3,5-$P_2$ Synthesis to Regulate Early Endosome Dynamics and Fusion**

O. C. Ikonomov, D. Sbrissa, A. Shisheva; Physiology, Wayne State University, Detroit, MI

Perturbations in the intracellular PtdIns 3,5-$P_2$ pool or the downstream transmission of PtdIns 3,5-$P_2$ signals often result in a gradual development of gross morphological changes along the pleiomorphic multivesicular endosomes, culminating with the appearance of cytoplasmic vacuoles. To identify the onset of PtdIns 3,5-$P_2$ functional requirements along the endocytic system, we characterized here morphological changes associated with early expression of the dominant-negative kinase-deficient form (K1831E) of the PtdIns 3,5-$P_2$-producing kinase PIKfyve, prior to formation of cytoplasmic vacuoles in transfected COS cells. Enlarged PIKfyve$^{K1831E}$-positive vesicles colocalizing with dilated EEA1- and Rab5a$^{WT}$-positive perinuclear endosomes were observed. This was dependent on the presence of active forms of Rab5 and the generation of PtdIns 3-P-enriched platforms on early endosomes. Because PIKfyve$^{WT}$ did not substantially colocalize with EEA1- or Rab5-positive endosomes, the dynamic PIKfyve-catalyzed PtdIns 3-to-PtdIns 3,5-$P_2$ switch was suggested to drive away PIKfyve$^{WT}$ from early endosomes toward later compartments. Late endosomes/lysosomes marked by ectopically expressed LAMP1 or Rab7 were dislocated from their typical perinuclear position upon PIKfyve$^{K1831E}$ early expression. Cytosols derived from cells stably expressing PIKfyve$^{K1831E}$ stimulated endosome fusion *in vitro*, whereas PIKfyve$^{WT}$-enriched cytosols had the opposite effect, consistent with PtdIns 3,5-$P_2$ production negatively regulating the endosome fusion. Together, our data indicate that PtdIns 3,5-$P_2$ defines specific endosome platforms at the onset of the degradation pathway to regulate the complex process of membrane remodeling and dynamics.

1966

**Pacsin 1 Tetramers are Targeted to Vesicles**

M. Milbrandt,[1] A. Halbach,[1] M. Mörgelin,[2] M. Plomann[1]; [1]University of Cologne, Centers for Biochemistry and Molecular Medicine Cologne (CMMC), Cologne, Germany, [2]Lund University, Department of Cell and Molecular Biology, BMC, Lund, Sweden

In eukaryotic cells complex regulatory mechanisms involving numerous proteins must operate to ensure the temporal and spatial specificity of intracellular membrane-trafficking pathways. We previously have identified the three members of the PACSIN protein family, which participate in rearrangements of actin networks during endocytosis. The isoform-specific expression patterns implicate a more general role for PACSINs in membrane traffic events, most of which occur at membranes of intracellular sorting compartments and vesicles. PACSINs bind to proteins involved in vesicle endocytosis and transport and act as linkers between the endocytic protein dynamin and N-WASP, thereby directing the actin propulsion

machinery to the site of vesicle fission. According to our model, dynamin remains attached to a released vesicle membrane and serves as anchoring site for PACSIN monomers, which oligomerise, recruit the propulsion complex to these sites and induce actin polymerisation. To address this hypothesis we have performed a series of experiments using recombinantly expressed PACSIN 1 to gain more insights into the oligomerisation of this protein in solution. PACSIN 1 dimers and tetramers interact via their N-terminal regions in a parallel orientation and form a barrel-like structure. In vivo two subpopulations of PACSIN 1 exist in microsomal and cytosolic fractions. In agreement with our model PACSIN oligomers are associated with vesicle membranes and can be localized to single sites on the vesicle surface as determined by ultrastructural analysis of negatively stained specimens in combination with immunogold labelled antibodies. We propose that PACSIN oligomers are required for vesicle removal from donor membranes.

1967

**Fc-receptor neonatal (FcRn) Mediates IgG Transport from the Apical or Basolateral Membrane of Polarized Epithelial Cells Intersects within a Common Endosomal Compartment**

S. Tzaban, E. Yen, M. Kothe, J. S. Wagner, W. I. Lencer; GI/Nutrition, The Children's Hospital, Harvard Medical School, Boston, MA

FcRn binds and traffics IgG bidirectionally across mucosal epithelial barriers where IgG acts in immune surveillance. The mechanism(s) of transport across polarized epithelial cells are unknown. Here we use polarized MDCK-II cells, stably expressing wild type FcRn (wt) or FcRn fused to green fluorescence protein (FcRn-GFP) to define the itinerary of FcRn-mediated IgG transport across polarized epithelial cells that model mucosal surfaces. Polarized MDCK-II cells stably expressing FcRn-GFP exhibit basolateral polarity of FcRn at the cell surface and transport IgG bidirectionally across the monolayer with the same efficiency as MDCK cells expressing wild type FcRn. At steady state, FcRn co-localizes with the transferrin receptor and the early endosome antigen 1 (EEA1) but not LAMP-1a, suggesting the sorting of FcRn to either early or common endosomes. In living cells, the FcRn-vesicles are strictly sorted away from the lysosomal compartment labeled with LysoTracker Red. Following uptake from either the apical or the basolateral surfaces, fluorophore-labeled IgG always co-localizes with FcRn. In contrast, chicken IgY that cannot bind FcRn is not internalized by these cells at all. Apically and basolaterally applied IgG meet in an apically located compartment that always localizes with transferrin. These results suggest that both the basolaterally- and apically-directed transcytotic pathways may meet in the common endosomal compartment. Whether these findings point to a common pathway remains to be determined.

## Protein Folding & Assembly in the Endoplasmic Reticulum II (1968-1984)

1968

**The Functions of ERp57 in the Folding and Assembly of MHC Class I Molecules**

Y. Zhang, D. B. Williams; Biochemistry, University of Toronto, Toronto, ON, Canada

Major Histocompatibility Complex (MHC) class I proteins consist of three subunits: the heavy chain (H chain), which is a transmembrane glycoprotein; a small soluble subunit termed $\beta_2$-microglobulin ($\beta_2$m), and an 8-9 residue antigenic peptide. Folding and assembly of class I molecules require multiple coordinated intra- and intermolecular events within the endoplasmic reticulum. The association of the thiol oxidoreductase ERp57 with free MHC class I H chains and with the class I peptide loading complex has suggested that this enzyme may participate in H chain disulfide formation/isomerization as well as in the loading of high affinity peptides. Here we present *in vivo* evidence that ERp57 does indeed participate in oxidative folding of the class I heavy chain. Depletion of ERp57 by RNA interference delayed H chain disulfide bond formation, slowed folding of the H chain $\alpha_3$ domain and caused slight delays in the transport of class I from the endoplasmic reticulum to the Golgi apparatus. In contrast, H chain-$\beta_2$m association kinetics were normal, suggesting that the interaction between H chain and $\beta_2$m does not depend on an oxidized $\alpha_3$ domain. Likewise, the peptide loading complex assembled properly except for the absence of ERp57. Peptide loading also appeared normal in the absence of ERp57 as evidenced by unaltered stability of cell surface class I molecules and by the normal production of complexes between the H-2K$^b$ molecule and the ovalbumin peptide, SIINFEKL. These studies constitute the first direct demonstration that ERp57 is involved in disulfide formation *in vivo* but do not support a role for ERp57 in peptide loading of class I molecules. Interestingly, depletion of another thiol oxidoreductase, ERp72, had no detectable effect on class I biogenesis, consistent with a specialized role for ERp57 in this process.

1969

**The FK506 Binding Protein Fkbp13 Interacts with and Stabilizes Presenilin**

D. L. van de Hoef, H. Wu, Y. Liu, G. Boulianne; Developmental Biology, Hospital for Sick Children, Toronto, ON, Canada

Presenilins were identified as causative factors in Familial Alzheimer's disease and also play an essential role in Notch signalling during development. Presenilins function in the multi-molecular γ-secretase complex, which cleaves type 1 transmembrane proteins including Notch and the beta-amyloid precursor protein (APP). To gain further insight into the function of Presenilins, we searched for *presenilin* interacting genes in *Drosophila*. Here we show that loss-of-function mutations in *Fkbp13* suppress phenotypes resulting from overexpression of *presenilin.* We also find that Fkbp13 binds directly to Presenilin and that Presenilin protein, but not RNA, is reduced significantly in *Fkbp13* null mutants. Finally, we show that FK506, which binds Fkbp13, also reduces Presenilin levels in addition to another component of the complex, Pen-2, thereby reducing γ-secretase assembly and activity *in vivo.* Together, our data demonstrate that Fkbp13 is an essential component of the Presenilin pathway. We propose that Fkbp13 is required to stabilize Presenilin protein allowing for formation of a functional γ -secretase complex.

1970

**Fluorescence-Detected Maintenance of the ER Membrane Permeability Barrier during the Co-Translational Integration of a Multi-Spanning Membrane Protein**

C. G. Jongsma, A. E. Johnson; Texas A&M University, College Station, TX

How is the permeability barrier maintained during the co-translational integration of a multi-spanning membrane protein (MSMP) at the translocon in the endoplasmic reticulum (ER) membrane? Previous work with a single-spanning membrane protein has shown that the aqueous translocon pore is sealed on the cytoplasmic side of the membrane by an ion-tight ribosome-translocon junction when the nascent chain is being transported into the ER lumen, and that this junction is opened for release of a nascent chain loop into the cytosol. However, junction opening does not occur

until the other end of the pore is first closed by the action of BiP after the transmembrane segment (TMS) of the nascent chain folds into an α-helix (or nearly so) far inside the ribosome nascent chain exit tunnel. The accessibility of the nascent chain to each side of the membrane was determined using cytosolic or lumenal iodide ions to collisionally quench the fluorescence of a probe incorporated into the nascent chain. Since the non-TMS segments of an MSMP are alternately directed into either the cytosol or the ER lumen, one would expect the nascent chain to alternate in its exposure to cytosolic or lumenal iodide ions as one TMS after another of a MSMP moves through the exit tunnel. Iodide ions were therefore added to fluorescent intermediates with different nascent chain lengths. Our studies reveal that an open ribosome-translocon junction is closed when the next TMS is synthesized by the ribosome. The length of nascent chain between the two TM segments appears to alter only the timing of the closure. (Supported by NIH grant GM26494 and the Welch Foundation.)

1971

### Proteomics and Pharmacology of CFTR Intracellular Interactions

A. V. Koulov,[1] X. Wang,[1] W. A. Kellner,[1] J. A. Coppinger,[1] J. R. Yates,[1] W. E. Balch[1,2,3]; [1]Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Molecular Biology, The Scripps Research Institute, La Jolla, CA, [3]The Institute for Childhood and Neglected Disease, La Jolla, CA

Cystic fibrosis (CF) is the most common hereditary disease in the Caucasian population, caused by mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene. From over 1000 mutations identified, deltaF508 in the NBD1 cytosolic domain of the protein is by far the most prevalent one, leading to folding defects and subsequent failure of the protein to engage the ER export machinery. To interrogate the direct interactions of CFTR within the export pathway, we have utilized mass-spectrometry-based multi-dimensional protein identification technology (MuDPIT). The focus of this study is to elucidate the interactions of CFTR with the protein machinery that couple folding to export. Using such a proteomic approach we have examined the role of Hsp90 and a dynamic complex of co-chaperones in the ER export of CFTR in normal and a disease state. Characterization of the effects of novel small molecules that modulate ER folding and export of mutant CFTR is anticipated to lead to discovery of new targets for future therapies.

1972

### Interaction of Wild Type and Mutant Human $Cl^-/HCO_3^-$ Anion Exchanger 1 with Molecular Chaperones

S. T. Patterson, D. B. Williams, R. A. F. Reithmeier; Biochemistry, University of Toronto, Toronto, ON, Canada

Anion Exchanger 1 (AE1, or Band 3) is an abundant red blood cell polytopic membrane glycoprotein responsible for the exchange of chloride and bicarbonate ions across the membrane as well as the anchoring to the cytoskeleton. Numerous point mutations and deletions have been identified leading to a loss of function as well as retention of AE1 in the endoplasmic reticulum (ER) during biosynthesis. Co-immunoprecipitation as well as peptide arrays were used to examine the interaction of AE1 with various chaperones. AE1 was expressed in a cell-free translation system using rabbit reticulocyte lysate supplemented with canine pancreatic microsomes and co-immunoprecipitated with antibodies to several chaperones. Wild type AE1, as well as the R760Q Hereditary Spherocytosis mutant and the Southeast Asian Ovalocytosis deletion mutant (Δ400-408) were shown to interact with the ER chaperones calnexin, calreticulin, and BiP, as well as the cytosolic chaperones Hsp70 and Hsp90. AE1 and the R760Q and SAO mutants were also expressed in HEK 293 cells and radiolabelled using [$^{35}$S]-methionine. Wild type and the mutant forms of AE1 were found to co-immunoprecipitate with calnexin, and pulse chase experiments showed a prolonged interaction of the mutants with calnexin. A peptide array generated using SPOT synthesis consisting of peptides corresponding to the various cytosolic and extracellular loops of AE1 was also used to study the binding of calnexin and calreticulin. Both chaperones were found to bind preferentially to the third and fourth extracellular loops. These results demonstrate the importance of molecular chaperones in the biosynthesis of AE1 and the retention of misfolded mutants in the ER. (Supported by the CIHR Strategic Training Program in the Structural Biology of Membrane Proteins Linked to Disease).

1973

### Folding, Assembly and ER Export of Yor1p, a Yeast ABC Transporter Homologous to Human CFTR

J. Barry, L. Kung, M. Dorrington, M. C. S. Lee, E. A. Miller; Biological Sciences, Columbia University, New York, NY

Eukaryotic cells dedicate significant resources to the deployment of proteins to the membrane compartments that comprise the secretory pathway. Cells must strike a precise balance between ensuring that only fully folded and properly assembled proteins are allowed to leave the endoplasmic reticulum (ER) and in avoiding the accumulation of misfolded proteins within the ER lumen, which can equally have detrimental consequences. We study the close relationship between protein folding and packaging into ER-derived transport vesicles using a model membrane protein, Yor1p, which functions as a plasma membrane drug pump in *Saccharomyces cerevisiae*. We have characterized two cytoplasmic di-acidic motifs that function as ER export signals and facilitate Yor1p capture into COPII vesicles. Mutations in these motifs cause ER retention without affecting the folding of Yor1p. In contrast, mutation of a conserved Phe residue (analogous to the predominant mutation associated with human cystic fibrosis) causes Yor1p to misfold, precluding its capture into COPII vesicles. We have undertaken several approaches to identify cellular components involved in the quality control of forward protein transport through the secretory pathway. Several of these quality control mutants show an improved ability to package the misfolded mutant of Yor1p into COPII vesicles. In some cases, mutant cells are better able to fold Yor1p and this improvement is linked to the ability to mount the unfolded protein response (UPR). Our results suggest that activation of the UPR can improve the ability of cells to handle misfolded proteins and suggest novel therapeutic targets for cystic fibrosis drug development.

1974

### Calnexin and Calreticulin Facilitate the Folding of ABCG5/G8 Sterol Transporter Leading to Increase the Cell Surface Expression

T. Kouno,[1] T. Okiyoneda,[1] A. Niibori,[1] K. Harada,[1] H. Kusuhara,[2] T. Sugiyama,[1] T. Shuto,[1] M. S. Suico,[1] Y. Sugiyama,[2] H. Kai[1]; [1]Molecular Medicine, Faculty of Pharmaceutical Sciences, Kumamoto, Japan, [2]Graduate School of Pharmaceutical Sciences, Tokyo Univ, Tokyo, Japan

ATP-binding cassette (ABC) G5 (G5) and ABCG8 (G8) hetero-dimerize and function as a sterol transporter that limits intestinal absorption and promotes biliary excretion of neutral sterols. Both G5 and G8 are N-glycosylated in the endoplasmic reticulum (ER) and interact with a lectin-like chaperone, calnexin, which is thought to facilitate the folding of G5/G8 heterodimer. To further investigate the role of calnexin in the biogenesis of G5/G8 heterodimer, we examined the effect of calnexin overexpression and knockdown on the biogenesis of G5/G8. Our results reveal that calnexin overexpression stabilized the immature forms of both G5 and G8 leading to the increase of G5/G8 heterodimer at the plasma membrane.

Moreover, a calnexin homologue, calreticulin, also stabilized the immature forms of both G5 and G8. Interestingly, calreticulin significantly increased the cell surface expression of G5/G8 heterodimer. Knockdown of either calnexin or calreticulin decreased the immature forms of both G5 and G8 leading to the decrease in expression of G5/G8 heterodimer. These results demonstrate that calnexin and calreticulin stabilize the immature forms of both G5 and G8 in the ER and facilitate the hetero-dimerization leading to the increase of cell surface expression

1975

**The Role of Key Amino Acid Residues in *Staphylococcal* Nuclease Folding**

H. M. Chen, J. M. Wu, H. J. Fang; Institute of BioAgricultural Sciences, Academia Sinica, Taipei, Taiwan Republic of China

The role of key amino acid residues in *staphylococcal* nuclease folding H. M. Chen, J.-M. Wu and H.-J. Fang Institute of BioAgricultural Sciences, Academia Sinica, Taipei, Taiwan 115 R.O.C. *Staphylococcal* nuclease is a single domain protein with 149 amino acids. It has no disulfide bonds that make it a simple model for the study of protein folding. In this study, twenty mutants of this protein were generated each with a single base substitution of Gly for negatively charged Glu or Asp. The results using differential scanning microcalorimetry in thermal denaturation experiments, we identified two mutants, E75G and E129G, having approximately 43% and 44%, respectively, lower $\Delta H_{cal}$ values than the wild-type protein. Multiple interactions around E75 and E129 may generate a local area of stability. Furthermore, two mutants, W140A and K133A, and two truncated mutants, $SNase_{1-139}$ (W140O) and $SNase_{1-141}$ (E142O), were generated. Replacing Trp at 140 with an alanine (W140A) had greatly reduced secondary structure and conformational energy compared to the wild-type protein. Similar experiments with $SNase_{1-139}$ confirmed the unusually large contribution of the W140 to the stability and the structural integrity of protein. Based on these observations, this protein stability relies not only on those local stable segments formed by specifically charged amino acids, but also on the amino acid Trp at position 140.

1976

**Analysis of Knock-in Mice Expressing a Mutant BiP with the Retrieval Sequence Deleted**

M. Kashio,[1] N. Mimura,[2] K. Kimura,[2] T. Aoe[1]; [1]Anesthesiology, Chiba University Graduate School of Medicine, Chiba, Japan, [2]Medicine and Oncology, Chiba University Graduate School of Medicine, Chiba, Japan

The endoplasmic reticulum (ER) provides a folding environment where secretory and transmembrane proteins interact with chaperones. Some membranous imperfect proteins are statically retained in the ER, while other membranous and soluble ones require transport to the Golgi complex and retrieval for ER quality control. BiP is a member of the Hsp70 family of proteins and is one of the most abundant ER chaperones, assisting in protein translocation, folding and degradation. When secreted from the ER in the company of imperfect proteins, BiP is recognized with a carboxyl terminal Lys-Asp-Glu-Leu (KDEL) sequence and retrieved by the KDEL receptor from the post-ER compartment. Yeast studies show that the deletion of retrieval motif from BiP/Kar2 is dispensable. Thus, the significance of the post-ER compartment for quality control in a single cell is unclear. In order to investigate the physiological significance of BiP and its retrieval in multi-cellular organisms in vivo, we established knock-in mice expressing a mutant BiP with the retrieval sequence deleted by homologous recombination.The embryonic fibroblasts derived from the homozygous mutant embryo expressed the mutant BiP instead of the wild-type and grew apparently as well as the wild ones. However, the homozygous mutant BiP mice were died early after birth, indicating that the retrieval of BiP is essential for surviving of mammals.

1977

**The Physiologic Deficits of Aging May Be Due to Declines in the Concentrations of the Endoplasmic Reticulum (ER) Chaperones**

J. L. Holtzman, R. R. Erickson, L. M. Dunning; Pharmacology, University of Minnesota, Minneapolis, MN

**Background:** In eukaryotes the ER chaperones are highly conserved proteins that catalyze the posttranslational processing of all secretory and membrane proteins. Recent studies from our laboratory have suggested that declines in the ER chaperones and N-glycosylation may be the central, biochemical defects underlying Alzheimer's disease. We propose that similar declines in the ER chaperones could account for some of the physiological deficits associated with aging in other organ systems. **Methods:** A single batch of rats were maintained in a "state-of-the-art" facility from age 21 days to death. At regular intervals 6 animals were removed, killed by $CO_2$, and hepatic microsomes prepared. At the end of the study, 6 ER chaperones were determined by immunoblotting. **Results:** After reaching maturity liver weights and total microsomal protein content did not change with age. On the other hand, constitutive levels of ERp55, ERp57, ERp72, BiP and calnexin declined 30-50% with age. Calreticulin was unaffected. BiP, ERp55 and ERp57 showed marked swings with peaks occurring in midwinter and midsummer. The heights of these peaks declined 73% with age. **Conclusions:** ER, posttranslational, protein processing is critical for cell function and survival. It appears to be capacity limited, especially for large, intrinsic membrane proteins, such as the CFTR. Hence, our data on the effect of aging on ER chaperone content even in a relatively well conserved organ, the liver, lend support to our proposal that the loss of ER chaperones may be a major factor in the physiological declines observed with aging. This loss would appear to be specific for ER chaperones, since others have reported that cytosolic chaperones, such as HSC70, are unaffected by age.

1978

**Consequences of Aging and Oxidative Stress on Endoplasmic Reticulum Protein Quality Control Machinery**

Z. Cabartova, E. L. Snapp; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Misfolded transmembrane and secretory proteins are a feature of several age-associated diseases. The translocation of proteins into the endoplasmic reticulum (ER) and folding are mediated by the translocon and ER chaperones. Oxidative damage of some chaperones in aged tissues suggests folding machinery dysfunction contributes to age-related protein misfolding. We are examining three age-related changes in the protein translocation and folding machinery. First, we are biochemically characterizing changes in ER chaperone and translocon component levels and oxidative damage to components in aging mouse liver. Second, we are mimicking these changes in cultured cells to assess consequences of oxidatively damaged chaperone and translocon component functionality. Finally, we are using the tissue culture system, coupled with biophysical imaging techniques, to investigate how oxidative damage affects the organization and dynamics of the translocon and lumenal chaperone complexes. By characterizing the age-related changes in the machinery responsible for the biogenesis of membrane and secreted proteins, we hope to gain new insights into the relationship between oxidative damage and ER function during aging.

1979

**Dynamic Interconversion of Proteasome Populations Identified by 2D BN-PAGE/SDS-PAGE**

T. Shibatani,[1] E. J. Carlson,[1] A. L. McCormack,[2] K. Früh,[2] W. R. Skach[1]; [1]Biochemistry, Oregon Health & Science University, Portland, OR, [2]Vaccine and Gene Therapy Institute, Oregon Health & Science University, Beaverton, OR

The proteasome is the major enzyme involved in proteolysis of cytosolic, nuclear, and misfolded ER proteins. Using 2D BN-PAGE/SDS-PAGE, we developed a single-step purification scheme which isolated 5 distinct proteasome populations in cytosolic extract. MS/MS analysis of 2D gel bands identified these complexes as 20S, single-capped and double-capped PA28-20S, hybrid PA28 and PA700 capped proteasome, and 26S proteasome. All populations were present in untreated cytosol. ATP depletion completely eliminated PA700 cap binding to the 20S core as reported previously, but had no discernable effect on PA28 interactions. In contrast, inhibition of 20S β subunit activity with MG132 stimulated formation of double capped PA28-20S core and hybrid proteasomes containing both PA28 and PA700 caps. Formation of double-capped PA28-20S proteasomes and hybrid proteasomes occurred quickly and was completed after 60-minute incubation at 37$^{o}$C with concomitant decrease of free 20S particles. *clasto*-Lactacystine β-lactone also stimulated the PA28-20S association but to a lesser extent than MG132. Interestingly, in the presence of MG132, 26S and hybrid proteasomes as well as single-capped and double-capped PA28-20S proteasomes remained stably associated with partially degraded products of an ER-associated degradation substrate. Our results demonstrate that BN-PAGE provides a powerful tool to isolate cytosolic proteasome populations. Proteasomes can exchange caps rapidly in response to cytosolic conditions. Furthermore PA28-20S proteasome, and hybrid proteasome populations preferentially accumulate when β-subunit activity is compromised, indicating that the PA28 might participate in diverse functions in addition to antigen presentation.

1980

**Targeting E2 Ubiquitin-Conjugating Enzymes for Degradation by the Proteasome: Mechanism and Implications in Protein Quality Control**

T. Ravid, M. Hochstrasser; MB&B, Yale University, New Haven, CT

Newly synthesized secretory proteins and endoplasmic reticulum (ER) resident proteins are subject to quality control mechanisms that verify proper folding prior to transport to their site of activity. Key players in this sorting system are components of the ubiquitin-proteasome system (UPS) that reside in the ER and covalently tag misfolded proteins with polyubiquitin chains, allowing retrotranslocation and degradation by the proteasome. Many mechanistic features of the UPS in the ER remain poorly understood. Our recent studies provide new insights into the regulation of ER-associated E2 enzymes of the ubiquitin system. Genetic studies in the yeast *Sacchromyces cerevisiae* identified several degradation pathways in the ER, which exhibit distinct substrate specificities. We had previously demonstrated that the E2 enzymes Ubc6 and Ubc7, together with the E3 Doa10, mediate the degradation of a subset of substrates that are either soluble nuclear or ER membrane proteins. We find that these E2 enzymes form a stable degradation complex with Doa10 at the ER membrane. Interestingly, both Ubc6 and Ubc7 are subject to degradation by the proteasome. However, whereas Ubc6 is degraded in the ER by a Doa10-dependent pathway, Ubc7 is ubiquitinated in a soluble cellular compartment by a distinct set of ubiquitinating enzymes. Structure-function analyses of the E2 enzymes demonstrate that the conserved cysteine in their active sites is essential, but their E2 catalytic activity is dispensable for degradation of the proteins. This implies that besides transferring ubiquitin to a substrate, the conserved cysteine in the E2 active site also plays an important role in the regulation of E2 enzyme degradation. The physiological function of this unique mechanism for ER-associated protein degradation and its mechanistic details are currently under investigation and will be discussed.

1981

**Heat Shock Protein 70-Interacting Protein (Hip) from Cold-Adapted and Sub-Tropical Teleosts**

S. Hann, H. Detrich; Biology, Northeastern University, Boston, MA

For Antarctic marine ectotherms, the cold Southern Ocean (-2 to +2°C) poses energetic challenges to protein folding. To help elucidate mechanisms that maintain efficient protein folding at low temperatures, we have initiated a comparative structural and functional analysis of Hip, the Hsp70/Hsc70-interacting, multi-motif cochaperone (39-45 kDa) that plays important roles in the biogenesis, transport, and degradation of cytoplasmic proteins. *hip* cDNAs were isolated from kidney or spleen of two Antarctic fishes, *Notothenia coriiceps* and *Chaenocephalus aceratus*, and from the kidney of the sub-tropical zebrafish, *Danio rerio*. The deduced Hip proteins were highly conserved both in amino acid sequence and in modular structure. However, one motif, the GGMP repeat (found in 5-6 copies in mammals), was present in 11-12 copies in the Antarctic fish Hip but only 1-2 in zebrafish Hip. Because GGMP repeat length is the major feature that differentiates the fish Hip proteins, we propose that this region is important for thermal modulation of Hip function. To test this hypothesis, we expressed recombinant wild-type *N. coriiceps* and *D. rerio* Hip and examined the temperature dependence of thermal denaturation and of self-oligomerization of the proteins. Wild-type *D. rerio* Hip melted with a $T_m$ of 49-50 $^{o}$C (monitored by CD at 222 nm), whereas *N. coriiceps* Hip was less stable ($T_m$ = 39-40 $^{o}$C). Denaturation analysis of GGMP-deletion variants of Hip should clarify the role of this motif in thermal stability. Preliminary analysis of wild-type *N. coriiceps* and *D. rerio* Hip by analytical ultracentrifugation suggests that the former self-associates to form a dimer whose association constant is greater than that of the latter. We conclude that the GGMP motif may be important for tailoring Hip function to environmental temperature. Supported by NSF grants OPP-9815381, -0089451, and -0336932 to HWD.

1982

**Gene Expression and Phylogenetic Relationships Reveal a Complex Protein Disulfide Isomerase Family**

N. L. Houston,[1] T. Yang,[1] Q. Xiang,[1] R. Jung,[2] R. S. Boston[1]; [1]Botany, NCSU, Raleigh, NC, [2]Pioneer Hi-Bred International, A DuPont Company, Johnston, IA

Protein disulfide isomerases (PDIs) catalyze the formation of proper disulfide bonds and participate in ER quality control as part of the cellular ER-stress response. To identify plant PDI-like (PDIL) proteins, we performed a genome-wide search of *Arabidopsis thaliana* and identified 104 thioredoxin (TRX) domain containing proteins, 22 of which group with orthologs of known plant PDIs in a well-supported clade. Using the Arabidopsis PDIL sequences in iterative BLAST searches, we identified orthologous sets of plant PDIL sequences in rice (19) and maize (22) and resolved the phylogeny into 10 classes. Five classes (I-V) had the two TRX domains typically found in PDIL proteins in other higher eukaryotes

while the remaining five classes (VI-X) had a single TRX domain. RNA profiling and quantitative RT-PCR analyses of the maize PDILs (ZmPDILs) showed marked differences in expression within and among classes. The major PDI (ZmPDIL1-1), the class V PDIL (ZmPDIL2-3), and the class VI maize PDIL (ZmPDIL5-1) were up-regulated by ER stress. Subcellular fractionation and immunoblot analysis revealed that ZmPDIL1-1 but not ZmPDIL5-1 was localized to the intracellular membrane fractions. ER-stress induction in the absence of ER localization is suggestive of an unusual role for the PDIL5-1 family member.

1983

**Role of Hsp90 in the Exocytic Pathway**

P. G. LaPointe, C. Gurkan, W. E. Balch; Molecular and Cell Biology, The Scripps Research Institute, La Jolla, CA

Packaging of protein cargo molecules into COPII vesicles at the ER is regulated through a balance between folding, cargo selection and degradation. Several lines of evidence suggest that the Hsp90 chaperone system contributes to cargo folding pathways including the cystic fibrosis transmembrane conductance receptor (CFTR). Our objective is to identify and characterize the role of the Hsp90 chaperone system in protein folding and ER export. Using yeast two-hybrid screens and proteomics we have identified Hsp90 co-chaperones involved in ER export. One of these is the conserved protein, Aha1, which has been previously been shown to interact with Hsp90 and accelerate its ATPase activity. We now report that hAha1 is itself a novel GTPase/ATPase with a peptide binding activity. Moreover, these two activities localize to two distinct domains of the protein. siRNA depletion of hAha1 results in a marked increase in the secretion of protein cargo proteins as well as an increase in ER stability of misfolded cargo molecules including the Δ508 variant of the CFTR protein. Our results suggest that hAha1 is potentially involved in regulating the presentation of client molecules to the Hsp90 complex for folding and export.

1984

**Plant UBX-domain Containing (PUX) Proteins Regulate the Function of *Arabidopsis* CDC48**

D. M. Rancour, S. Park, S. Y. Bednarek; Biochemistry, University of Wisconsin-Madison, Madison, WI

CDC48/p97 is a conserved and essential hexameric AAA-ATPase that functions as a molecular chaperone in numerous diverse cellular activities. CDC48/p97 activity is recruited to specific functions through its interaction with adapter proteins. Our working hypothesis is that AtCDC48 and the *Arabidopsis* homolog of syntaxin5/Sed5p, SYP31, mediates events important for plant cytokinesis. Using affinity chromatography and MALDI-TOF mass spectrometry we have identified two uncharacterized plant UBX-domain containing proteins, PUX1 and PUX2, which interact with AtCDC48. UBX-domains are ubiquitin-like protein folds that function as interaction domains for CDC48/p97. PUX1 is a soluble protein that co-fractionates with non-hexameric AtCDC48 from plant cell extracts and inactivates AtCDC48 through hexamer disassembly. The UBX C-terminus of PUX1 contains the primary determinants necessary for interaction with AtCDC48. Two null insertion mutant alleles of PUX1 have been identified and show no gross morphological abnormalities, however, these plants surprisingly display accelerated growth rates. We propose that PUX1 elicits its negative effect on growth by promoting the inactivation of AtCDC48 function *in vivo*. In contrast to PUX1, PUX2 is a peripheral membrane protein that interacts with AtCDC48 *in vitro* and co-fractionates with membrane-associated but not soluble AtCDC48 *in vivo*. Biochemical reconstitution and immunolocalization data suggested that PUX2 facilitates the interaction of SYP31 and AtCDC48 during interphase and cytokinesis, thereby regulating an AtCDC48 membrane-associated function. Deletion analysis of PUX2 protein domains revealed the requirement of its PUG domain and not its UBX-domain for interaction with AtCDC48. Sequence analysis of PUX2 suggested that divergence in amino acid composition of the UBX-domain might be responsible for the inability of the domain to interact with AtCDC48. These studies provide the first evidence that the PUG domain may be an alternate interaction domain for AtCDC48.

## Tissue-Specific Gene Expression (1985-2002)

1985

**Human Dental Pulp Cell Culture and Cell Transplantation Using Alginate Scaffold**

S. Kumabe, F. Aikawa, M. Nakatsuka, H. Imbe, Y. Iwai; Oral Anatomy, Osaka Dental University, Hirakata-shi, Japan

[Purpose] We research stem cells in human dental pulp (HDP), because our preliminary study observed that cultured HDP cells induced hard tissue formation. The present study examines the transplantation of cultured cells using alginate as a scaffold.[Materials & Methods] The HDP tissue was obtained from extracted tooth, and cultured 13 passages. We added beta-glycerophosphate to examine cell differentiation, and examined ALPase activity related with tissue mineralization. Then, for studying differentiation of odontoblast, the expression of dentin sialophosphoprotein (DSPP) mRNA was studied by reverse transcriptase polymerase chain reaction (RT-PCR). For the investigation of the effect of scaffold, we mixed cultured cells and 1.5% alginate solution to form a cell cluster, which was then transplanted to the subcutaneous layer of 7-week old immunodeficient mice. Super soft X-ray (SOFTEX) films were taken to identify mineralization of the transplanted cell cluster. After 6 weeks, the tissue was removed, embedded in paraffin and type I & III collagen was immunohistochemically studied. Fine structure of the tissue was studied by the conventional TEM. [Results] Fibroblast-like cells were observed in the 13th passage samples. Diachronic increase of ALPase activity and expression of DSPP mRNA were increased by adding beta-glycerophosphate. Mineralized nodules were observed in cultured cells. Formation of hard tissue was observed within the transplanted cell cluster using alginate scaffold. Type I & III collagen antigen reaction was observed in the mineralized tissue surrounded and interposed with many fibroblast-like cells. [Discussion] The 13th passage cultured cells differentiated odontoblast-like cells. Expression of DSPP mRNA and formation of X-ray opaque tissue containing type I & III collagen indicated that transplanted cells commenced formation of certain dentin-like substance. [Conclusion] The present study suggests that alginate is an effective scaffold for dental pulp cells to produce dentin-like tissue.

1986

**Optimization of Spider Silk Glands for the Elicited Production of a Tissue-Specific Fibroin Product**

A. Garcia, I. Cajigas, B. Marrero, X. Cajigas, S. Medina, J. Rodriguez, G. Arroyo, G. C. Candelas; Biology, University of Puerto Rico, San Juan, Puerto Rico

Our studies have revealed that fibroin production by the large ampullate glands of the spider *Nephila clavipes* is the culmination of a series of time

and tissue-specific events, which optimize the glands for their highly demanding function. The latter is attained through a series of transient bouts of activities which enrich the gland's protein synthesis machinery. The processes are reproducible in terms of their timing and also relative intensities. The earliest of these events, one of considerable magnitude, provides the glands with a variety of small RNAs: two isoforms of 5S rRNA, a tissue-specific alanine tRNA, and members of the U subset of small RNAs which constitute the spliceosome complex. Isoforms of U1 and U2 generated have been proven not to be intermediates of RNA processing. The second event, which generates the fibroin template, is followed by an event of rather limited magnitude which attains selective enrichment of the gland's tRNA population by generating the tRNAs cognate to the fibroin's predominant amino acids: glycine, alanine and proline; an adaptive tRNA population shift. The last of the transient events, and one of considerable magnitude, attains the culmination of the elicited process through the massive production of the full size fibroin. Of special interest is the generation of a tissue-specific alanine tRNA of considerable intensity. Although its function is not, as yet, known it is a feature in the fibroin production strategy of the silkworm *Bombyx mori.*

1987

**Detection of HLA-E and HLA-G Expression in Human Placental Tissue**

H. Hutter, M. Siwetz, A. Blaschitz, G. Dohr; Institute of Cell Biology, Histology and Embryology, Medical University of Graz, Graz, Austria

Nonclassical MHC class Ib human leukocyte antigen E (HLA-E) and HLA-G molecules differ from classical ones by specific patterns of transcription, protein expression, and immunotolerant functions. HLA-G can be expressed as four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) proteins upon alternative splicing of its primary transcript. In this study, we used a set of monoclonal antibodies (mAbs) called HCA2, 4H84, MEM-G/1, -G/9, MEM-E/2, and -E/6 recognizing HLA-G or HLA-E. The patterns of reactivity of these mAbs were analyzed on transfected cells by Western blotting and immunocytochemistry. MEM-G/1 recognizes (similar to the 4H84 mAb) the denatured HLA-G heavy chain of all isoforms. MEM-G/9 mAb react exclusively with native HLA-G1 molecules. MEM-E/2 and -E/6 mAbs bind the denatured and cell surface HLA-E molecules, respectively. These mAbs were then used to analyze the expression of HLA-G and HLA-E on cryo-preserved and paraffin-embedded serial sections of placental tissue. Single and double-immunolabeling with the respective mAbs revealed the presence of HLA-G and HLA-E in extravillious cytotrophoblast. In comparison, however, the levels of HLA-E expression seem to be much lower than those of HLA-G. In addition, we found a strong expression of HLA-E in endothelial cells using mAb MEM-E/2. The presence of HLA-G and HLA-E in specific cell populations of the placenta suggest an interacting functional role of these molecules in maternal-placental immune recognition.

1988

**Cdx2, Gata-4 and Hnf-4α Lead to Expression of an Intestinal Epithelial Phenotype in Fibroblasts**

J. Babeu, C. R. Lussier, F. Boudreau; Anatomy and Cell Biology, University of Sherbrooke, Fleurimont, PQ, Canada

The intestinal epithelium is an excellent model to study the molecular mechanisms during cellular differentiation. The epithelial stem cells located at the bottom of the intestinal crypts constantly divide to generate cells that will differentiate into four cell lineages. This differentiation is tightly regulated during cellular migration up to the villi. Literature supports the idea that restricted expression of many intestinal specific gene in differentiated cells is due to the specific combination of transcription factors. However, little is known about the exact molecular mechanisms that ultimately lead to intestinal epithelial cell differentiation. In the light of recent observations, we followed the hypothesis that Cdx2, Gata-4 and Hnf-4α could act as a transcriptional complex and induce cells to specialize into intestinal epithelial cells. To test this hypothesis, we first generated fibroblastic cell lines (NIH-3T3) that can conditionally over-express different combinations of Cdx2, Gata-4 and Hnf-4α transcription factors. We then characterized these inducible cell lines by Western blot analysis. NIH-3T3 cell lines that showed the best controlled expression for each factors were chosen to further investigate. RT-PCR analysis revealed that the combination of Cdx2, Gata-4 and Hnf-4α resulted into the induction of expression of the intestinal epithelium specific genes intestinal-fatty-acid-binding-protein (IFABP), intestinal trefoil factor 3 (ITF3) and apolipoprotein A-IV. We also found by electronic microscopy that NIH-3T3 with combined expression of Cdx2, Gata-4 and Hnf-4α changed morphology with the increase in cellular dimension, appearance of microvilli and beginning of polarization. In conclusion, our results suggest that ectopic combination of HNF-4α, Cdx2 and GATA-4 can initiate a program of intestinal epithelial cell determination within the mesenchymal context. Further studies will be necessary to document the targets of these transcription factors during the acquisition of the intestinal epithelial phenotype.

1989

**Functional Characterization of the Liver-enriched Transcription Factor CREB-H**

K. Chin,[1] J. Wong,[2] I. Ng,[2] D. Jin[1]; [1]Biochemistry, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region of China, [2]Pathology, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region of China

We have previously characterized transcription factor LZIP/CREB3 to be a growth suppressor targeted by hepatitis C virus oncoprotein. In search of proteins closely related to LZIP, we have identified a liver-enriched transcription factor CREB-H/CREB3L3. LZIP and CREB-H represent a new subfamily of bZIP factors. CREB-H activates transcription by binding to cAMP responsive element, box B, and ATF6/UPRE-binding element. Interestingly, CREB-H has a putative transmembrane domain and it localizes ambiently to the endoplasmic reticulum. Proteolytic cleavage that removes the transmembrane domain leads to nuclear translocation and activation of CREB-H. CREB-H activates the promoter of hepatic gluconeogenic enzyme phosphoenolpyruvate carboxykinase. This activation can be further stimulated by cAMP and protein kinase A. CREB-H transcript is exclusively abundant in adult liver. In contrast, the expression of CREB-H mRNA is aberrantly reduced in hepatoma tissues and cells. The enforced expression of CREB-H suppresses the proliferation of cultured hepatoma cells. Taken together, our findings suggest that the liver-enriched bZIP transcription factor CREB-H is a growth suppressor that plays a role in hepatic physiology and pathology.

1990

**Characterization of Two New Members of the *XK* Gene Family: XPLAC and XTES**

G. Calenda, J. Peng, C. M. Redman, Q. Sha, X. Wu, S. Lee; Membrane Biochemistry Laboratory, Lindsley F Kimball Research Institute of the New York Blood Center, New York, NY

XK, a putative membrane transporter, is a component of XK/Kell complex of the Kell blood group system. The substrate of XK is unknown but

absence of the protein results in neuromuscular abnormalities known as McLeod syndrome. We have cloned two XK homologues, *XPLAC* (AY589511) and *XTES* (AY989815), which form the *XK* gene family sharing 33% or higher amino acid identities. XK and XPLAC are present from human to fish. Gene linkage analysis demonstrates that *XK* is the primary copy from which *XPLAC* is duplicated. Currently *XTES* is found only in primates, suggesting *XPLAC/XTES*-splitting is the latest gene duplication event. Phylogenetic analysis revealed that the *XK* gene family is distantly related to at least 5 other vertebrate genes down to fish. However, less homologous proteins are present in nematodes and chordates. Ced-8 in *C. elegans* shares 19% amino acid identity with XK but it is more closely related to the 5 distantly related genes than to the XK gene family. Ced-8 has been reported to be involved in apoptosis (Stanfield, GM., Horvits,R., *Molecular Cell*. 2000; 5:423-433). Unlike ubiquitously expressed XK, XPLAC is mostly expressed in placenta and adrenal gland while XTES expression is limited to testis. Transfection of XPLAC and XTES cDNAs in COS-1 cells produced proteins of about 42 and 40 kDa, respectively, which are smaller than the predicted sizes of 53.6 (XPLAC) and 53.4 kDa (XTES). Immunohistochemistry of human placenta revealed that XPLAC is present in syncytiotrophoblasts. RT-PCR of a human trophoblast cell line, BeWo, showed the presence of XPLAC mRNA. Immunofluorescence detected XPLAC on the plasma membrane of BeWo cells. Cell surface biotinylation showed that XPLAC is on the plasma membrane of transfected COS-1 cells as well. Supported by NIH grants, HL54459 and HL075716

1991

**Aggrecan Upstream Regulatory Element P3 Directs Expression Exclusively to Maturing Chondrocytes**

K. Doege, F. Galiano; Pathology, LSU Health Sciences Center, Shreveport, LA

Aggrecan is a large chondroitin-sulfate proteoglycan expressed primarily in cartilage, where it is a major and essential component of the extracellular matrix. Stimulation of aggrecan synthesis is one of the goals in tissue engineering of cartilage, while depletion of aggrecan either by catabolic processes or altered cellular metabolism is one of the hallmarks of degenerative joint disease. Progress in understanding aggrecan gene regulation was recently achieved by identification of a regulatory element 12 kb upstream of the promoter which conferred cartilage-specific expression in transgenic mice. This element, (P3), appeared to direct expression to a subset of cartilages. The object of the current study was to determine the developmental course of expression driven by the P3 element, and relate it to the endogenous aggrecan gene expression pattern. Transgenic mice bearing the P3-driven lacZ transgene were bred, and litters were harvested at stages E14.5 and E16.5 p.c. The embryos were fixed and embedded in paraffin, and processed for in situ hybridization on tissue section using radioactive antisense cRNA probes. The transgene was monitored using a probe for the lacZ reporter, while other probes were also generated for aggrecan, type II collagen, type X collagen, Sox9, and Cbfa1. The results showed that aggrecan and type II collagen have very similar expression domains, while the P3-driven reporter was restricted to maturing cartilage of the lower proliferative/upper hypertrophic zone. This is the zone of highest expression for the endogenous aggrecan gene, and overlaps with type X collagen expression. Both Sox9 and Cbfa1 are expressed in this region also. These results confirm that the aggrecan regulatory apparatus is composed of combinatorial elements which may give restricted patterns of expression individually, but must be assembled together to reconstitute the entire endogenous expression pattern.

1992

**Identification of Two Repressor Elements Responsible for Cell-Specific Expression of an Osteoclastic Protein-Tyrosine Phosphatase (PTP-oc) Within its Intronic Promoter**

J. Yang, M. Amoui, K. H. W. Lau; Musculoskeletal Disease Center, J. L. Pettis Memorial VAMC, Loma Linda, CA

PTP-oc plays an important role in osteoclast functions. Although PTP-oc shares the same gene with a renal receptor PTP, GLEPP1, it is a distinct gene driven by a cell-specific, intronic promoter. To identify regulatory elements responsible for cell-specific expression of PTP-oc, we performed deletion and site-directed mutagenesis analyses on the pGL3-murine PTP-oc intronic promoter reporter construct. The pGL3-PTP-oc promoter construct showed strong basal promoter activity in osteoclastic cells, RAW264.7 cells, but was inactive in non-osteoclastic cells (TE85 osteoblastic cells, HepG cells, and skin fibroblasts). Deletion analyses performed with 13 pGL3-PTP-oc promoter deletion constructs identified two regulatory regions (-931/+116 and -491/+116, respectively). Internal deletion of each of these two regions from the promoter alone each led to >90% reduction in promoter activity. Simple alignment analysis between the human and mouse promoter sequences revealed no homology. When potential transcription factor binding regions (identified with the TESS program) of human promoter sequence was aligned against the murine promoter, at least 9 conserved domains were identified within the regulatory regions of the promoter. Site-directed mutagenesis analyses of each conserved domain revealed that at least two sites (potential binding site for IK-1/2 and IL6-RE-BP, respectively) contained repressor sites, since mutation of each site led to a significant increase in basal transcription activity in RAW264.7 cells. Importantly, each of the two mutated promoter constructs was equally active in non-osteoclastic cells as was in RAW264.7 cells, indicating that these two repressor sites were responsible for cell-specific expression of PTP-oc. In conclusion, we have identified two repressor elements responsible for cell-specific expression of PTP-oc within its promoter.

1993

**Multiparametric Gene Expression Profiling of Umbilical Cord Blood Multi Lineage Progenitor Cell Line**

N. Forraz,[1] M. Baradez,[2] C. P. McGuckin[1]; [1]Regenerative Medicine, Clinical Laboratory Sciences, University of Newcastle, Newcastle upon Tyne, United Kingdom, [2]Stem Cell Therapy Programme, Kingston University, Kingston upon Thames, United Kingdom

OBJECTIVES: Development of reproducible tissue engineering protocols calls for reliable access to sufficient numbers of immature homogeneous cell groups. High definition microarray analysis was used to phenotype the umbilical cord blood (UCB) cell line: Multi Lineage Progenitor Cell line (MLPC -BioE, MN, USA). Multiparametric gene expression analysis compared MLPCs to UCB mononucleated cells (MNCs), PrepaCyte-purified cells, CD133+ progenitor cells, lineage-restricted stem cells (Lin-) and a bone marrow mesenchymal stem cell (BMMSCs) in order to span a range of cell groups at various stages of differentiation. METHODS: RNA samples were hybridized to the PIQOR 942 gene Human Stem Cell Microarray platform (Miltenyi Biotec, CA, USA). MLPCs were compared to each cell group above with full pathway and signal network bioinformatics analysis. RESULTS: MLPCs phenotypic expression profile was significantly different (>1.4 fold down- or up-regulated) by 631 genes to the other five cell groups. MLPCs were particularly different to mature cell populations (MNCs and PrepaCyte) with downregulation of 65 genes associated with active protein synthesis (e.g. ribosomal sub-units), 18 genes linked with phosphate metabolism (kinases and phosphatases),

123 genes regulating proliferation and cell cycling (cyclins, cyclin-dependent kinases, check point proteins); reflecting a high degree of stemness, immaturity and quiescence. Comparison to stem and progenitor cell subsets (CD133+, Lin-) highlighted MLPCs' multipotential with downregulation of 12 different cluster of differentiation surface marker genes (e.g. epithelium, endothelium) and upregulation of 80 genes involved in nucleic acid binding and/or transcription factor activity regulating differentiation of tissues from all three germ layers. CONCLUSION: Stem cell-specific microarray phenotyping charaterized MLPC line as homogeneous group of extremely immature cells with exciting capabilities for tissue engineering applications.

1994

**Megakaryocytopoiesis and Zinc Finger Protein 24 (ZNF24)**

A. Hughes,[1] I. B. Sundell,[2] K. Tsubata,[1] J. Wooden,[1] J. A. Reems,[1] D. M. Gilligan[1]; [1]Puget Sound Blood Center, Seattle, WA, [2]Cellular and Medical Pharmacology, UCLA, Los Angeles, CA

Differentiation of megakaryocytes from hematopoietic stem cells is a complex process that occurs within human bone marrow. Megakaryocytes are large cells (50-70 micrometers) that are hyperdiploid (8N to 64N) due to incomplete cytokinesis. The final event of differentiation is platelet production, whereby each megakaryocyte produces hundreds of platelets. Given that the regulation of megakaryocyte differentiation and platelet production is not well understood, microarray analysis was performed on human hematopoietic stem cells (CD34+/CD38lo) that were induced to undergo megakaryocytic differentiation with thrombopoietin during a ten day culture period (Shim, et al., 2004). Among the genes identified to significantly increase in expression levels when comparing uncultured CD34+/CD38lo to day 10 culture derived cells was ZNF24. The mRNA level for ZNF24 showed an increase of 5.8$\pm$1.9 (mean$\pm$SEM; n=3) fold by microarray analysis, with a p-value of less than 0.001 (array performed in triplicate from three different cultures). Results were verified by real-time RT-PCR and analysis of hematopoietic lineages demonstrated lack of expression of ZNF24 in myeloid or lymphoid cells, with low levels of expression in erythroblasts and human hematopoietic stem cells cultured in the presence of erythropoietin. ZNF24 expression was also detected in cultures of murine megakaryocytes. We have expressed ZNF24 recombinant protein in E. coli and purified it using a 6xHis tag. Recombinant protein was used to generate an antibody in chickens and this antibody reacts strongly with a band of 50 kDa, the predicted size for the gene product. Confocal immunofluorescent microscopy demonstrates strong expression of ZNF24 in nuclei of human culture derived megakaryocytes. Since ZNF24 is a member of the large Kruppel-like family of transcription factors that play a variety of roles in regulating cellular differentiation, further studies will test the hypothesis that ZNF24 has a role in the regulation of megakaryocyte differentiation.

1995

**EST Analysis of Genes Expressed in Hemocytes of Manila Clams *(Ruditapes philippinarum)* Infected with *Perkinsus olseni***

S. K. Cho, Y. Kim, J. Kim, K. Choi, M. Cho; Cheju National University, Jeju, Republic of Korea

To investigate the patterns of genes expressed in Manila clams (*Ruditapes philippinarum*) infected with the protozoan parasite *Perkinsus olseni*, we constructed a cDNA library and generated 1,850 clones (expressed sequence tags). The 1,850 expressed sequence tags (ESTs) were compared to sequences in the GenBank database. Of these, 1,151 clones (62.2%) were unknown and are likely to represent newly described genes, while 699 clones (37.8%) were identified based on matches to sequences in the database. In all, 53 contigs and 376 singletons were formed. The identified ESTs could be grouped into seven functional categories: cell signaling/communication (14.9%), cell structure (9.0%), cell defense/immunity (11.3%), metabolism (17.9%), replication (1.9%), unknown functions (10.9%), and others (34.2%). In these categories, a total of 79 ESTs, such as C-type lectin, lysozyme, and cystatin B, were related to 29 functional immune genes in Manila clams infected with *Perkinsus olseni.* Lectins were the largest group of immune-function ESTs found in the Manila clams. A phylogenetic tree of these ESTs was constructed from a sequence alignment by the Jotun-Hein method (Megalign, DNAstar). The resulting phylogenetic tree showed that the Manila clam C-type lectin was more closely related to that of purple sea urchins than to that of scallops. The expressions of lectins in the hemocytes were analyzed by RT-PCR using gene specific primers. Hemocyte from *Perkinsus*-infected clam expressed different set of lectins from that of *Vibrio*-infected one. Here, we suggest various lectins are involved in Manila clam innate immunity and different challenges induce expression of a different set of lectins.

1996

**Possible Functional Consequences of Microgravity-Induced Changes in Gene Expression in Activated T-Lymphocytes**

N. E. Ward,[1] N. R. Pellis,[2] S. Risin,[3] D. Risin[2]; [1]Cell Science, Wyle Laboratories, Houston, TX, [2]NASA/JSC, Houston, TX, [3]UT Medical School, Houston, TX

Exposure to both true and modeled microgravity causes a decline in cellular immune function. The purpose of this study was to identify associated gene expression changes and possible gravity sensitive genes by applying microarray chip analysis. CD3- and IL2-activated T-cells were cultured in 1g (static) and modeled microgravity (NASA Rotating Wall Vessel bioreactor) conditions for 24 hours. Microarray analysis was performed utilizing Affymetrix Gene Chips that allow testing for 18,400 genes. Exposure to modeled microgravity resulted in altered expression of 89 genes, 10 genes showed up-regulation and 79 down-regulation. Up-regulated genes included IL7-receptor which plays a role in lymphocyte development and blocking of apoptosis during T-cell differentiation and activation. Also up-regulated were several heat shock protein family members functioning together as molecular chaperones, stabilizing existing proteins from aggregation, and mediating folding of newly translated proteins. Down-regulated genes involved in various roles in the induction of apoptosis included granzyme B, beta-3-endonexin, and Apo-2 ligand. Proteasome activator subunit 2, part of the immunoproteasome which processes MHC peptides was also down-regulated. Also down-regulated were three proteasome beta family members which cleave peptides, several genes potentially affecting the integrity of the cellular cytoskeleton such as CD2 associated protein, tubulin, and pericentrin 1, genes affecting DNA repair such as nudix and FEN1, and genes affecting several metabolic pathways such as glucose 6-phosphate dehydrogenase in the pentose phosphate pathway. (Supported by NRA OLMSA-02 and NSCORT NAG5-4072 grants).

1997

**Production Mechanisms of Plasma Glutathione Peroxidase from Bovine Adipocyte**

H. Aso,[1] T. Yamasaki,[2] K. Tahara,[2] S. Takano,[2] M. Inoue-Murayama,[3] T. Minashima,[1] T. Kanaya,[1] S. Ito[3]; [1]Cellular Biology Laboratory, Tohoku

University, Sendai, Japan, [2]R & D Center, BML Inc., Kawagoe, Japan, [3]Laboratory of Animal Genetics, Gifu University, Yanagido, Japan
Despite the well-known ability of glutathione peroxidases (GPx) to metabolize hydrogen peroxide, the exact role of this enzyme family under physiological and oxidative stress conditions is still not clearly defined. A plasma-secreted form of GPx (pGPx) is a member of this family, and kidney is a main producer of pGPx. We identified the large part of isolated genes as pGPx, which were subtracted the proliferating stage from the adipogenic differentiating stage of a bovine intramuscular preadipocyte (BIP) line. Therefore, we analyzed the production mechanisms of pGPx in bovine adipocyte. During adipogenic differentiation of BIP cells, the expression of pGPx genes was increased from 2 days and reached to the peak at 4 days after stimulation. The production of pGPx protein to the culture supernatant was detected at 6 days after stimulation and produced by dexamethason. The addition of octanate enhanced its production by dexamethason. In BIP cells, the peak of expression of C/EBP-gamma genes were about 6.4-fold at 2 days after stimulation, however, the expression of the GPx1 genes was hardly detected during adipogenesis. In 3T3-L1 cells and human preadipocytes, GPx1 gene was usually expressed at the proliferating and the differentiating phases. These data suggested that there might be a different production pathway of GPx between spices. The protein of pGPx was greatly produced in kidney and in intraperitoneal fat, but was weakly detected in lung, heart, spleen and small intestine. We considered that the transcriptional control of pGPx in ruminants might to be carried out by C/EBP-gamma, and that the expression of pGPx might be a characteristic phenomenon in bovine adipogenic differentiation.

1998

**Value of Microarray Analysis of Gene Expression for the Study of the Mechanism of Ischaemia/Reoxygenation in the Human Myocardium**

H. A. Vohra, M. F. Chowdhry, M. Galiñanes; Cardiovascular Sciences, Leicester University, Leicester, United Kingdom
OBJECTIVE We have previously shown that the degree of cell death by apoptosis and necrosis in the human myocardium depends on both the ischemic insult and the period of reoxygenation, that injury is exacerbated by diabetes and reduced by ischemic preconditioning (IP) and caspase-3 inhibition (C3i). Here we investigate changes in gene expression induced by ischemia (I) and reoxygenation (R) and the effect of cardioprotective interventions such as IP and C3i in the diabetic and non-diabetic human myocardium. METHODS Gene Chip ® microarrays were used to analyse mRNA isolated from right atrial appendage (n=3 specimens from different subjects/group) at the end of 90 minutes I and after 120 minutes R. Affymetrix Suite 4.0 and DNA-chip analyser 1.3 were used for data analysis. Changes in gene regulation were considered significant if hybridization intensity was more than 2-fold different from non-ischaemic controls. RESULTS Genes encoding for pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6, interferon-α) were down-regulated by I, whereas heat-shock proteins were unaffected by I but were up-regulated by R. A number of specific genes transcribing for pro- and anti-apoptotic proteins were down- or up-regulated, changes that may contribute to promote or counteract apoptosis. The Bcl-2 related gene was down-regulated by >6-fold by I and R but was attenuated by C3i (1.2-fold) and reversed by IP (3.1-fold up-regulation). Specific inflammatory, cell cycle, growth-related and cell death genes were up- or down-regulated by diabetes and I and R resulted in gene expression changes that were not observed in non-diabetics. CONCLUSION Use of microarray analysis and gene expression can be an important tool to understand the complexity of I and R injury, the role played by disease states such as diabetes and the mechanism of cardioprotection.

1999

**Nkx25 Knockdown Using RNAi Resulted in Sarcomere Disorganization**

H. Kasahara,[1] C. Martinov,[1] M. Marks,[1] H. Aoki[2]; [1]Physiology and Functional Genomics, University of Florida, Gainesville, FL, [2]Yamaguchi University, Ube, Yamaguchi, Japan
The homeobox transcription factor, Nkx2.5, is a well-characterized early cardiogenic marker in embryonic heart development. While Nkx2.5 expression continues in the heart throughout adulthood, its function in postnatal hearts is not well understood. We knocked-down Nkx2.5 using adenovirus-delivered RNAi in cultured rat neonatal cardiomyocytes. Depletion of Nkx2.5 expression (more than 90 %) was associated with altered cardiomyocyte cell shape with sarcomere disorganization. Expression of two Z-disc localized proteins, muscle LIM protein (MLP) and T-cap (titin cap protein), were markedly downregulated early after Nkx2.5 downregulation, suggesting that their expressions are dependent on Nkx2.5 expression. To understand the transcriptional regulation of the MLP gene, a 2kb promoter region of mouse MLP gene was analyzed in a luciferase reporter construct. Addition of this promoter region to pGL3-luciferase basic reporter vector caused a more than 50-fold induction of luciferase activity in cardiomyocytes compared to only 2- to 3-fold induction in non-myocytes (relative to pGL3 basic vector luciferase activity). These results indicate that this 2 kb proximal promoter region of the MLP gene contains strong cardiomyocyte-specific transcription factor binding sites. Five consensus Nkx2.5 binding sites are located in this region, which suggests direct involvement of Nkx2.5 on MLP gene expression. Our findings suggest that in the postnatal heart, Nkx2.5 is involved in transcriptional regulation of sarcomeric protein expression and maintenance or maturation of sarcomere organization.

2000

**New Technologies for Gene Expression Analysis from Blood**

P. Whitley, S. Motori, J. Kemppainen, J. Gonzoles, B. Setterquist, C. D. Buchanan, G. Latham; Ambion, Austin, TX
Blood is used extensively as a diagnostic tissue to indicate a variety of health problems including infection, inflammation, cancer and autoimmune disease. However, abundant globin transcripts in the blood greatly reduces array sensitivity and causes a progressive distortion of gene expression profiles in a manner that is dose dependent. Several cutting-edge technologies have converged to enhance the success of microarray experiments from blood. In particular, we have optimization sample stabilization, RNA isolation, globin mRNA reduction and RNA amplification from whole blood. These advances result in >95% removal of globin transcripts and increase the percent of detectable genes on Affymetrix arrays by more than 50%.

2001

**Methylation Profiling Using a Human Chromosome 1 Minimal Tiling Path Array**

E. Ramos,[1,2] C. De Bustos,[3] R. K. Tran,[4,5] U. Menzel,[3] S. McGrath,[2,5] C. F. Langford,[6] E. E. Eichler,[2,5] L. Hsu,[7] S. Henikoff,[4,5] J. P. Dumanski,[3] B. J. Trask[1,2]; [1]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA, [2]Department of Genome Sciences, University of Washington, Seattle, WA, [3]Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden, [4]Division of Basic Sciences, Fred

Hutchinson Cancer Research Center, Seattle, WA, [5]Howard Hughes Medical Institute, Seattle, WA, [6]Welcome Trust Sanger Institute, Cambridge, United Kingdom, [7]Division of Public Health, Fred Hutchinson Cancer Research Center, Seattle, WA

DNA methylation is a major epigenetic modification that plays an important role in gene expression and genomic imprinting. Aberrant methylation can lead to a number of diseases, including cancer. In mammals, some CpG islands, regions of dense CpG sequence, are involved in regulating transcription of many genes. Consequently, methylation at key CpGs can lead to the inhibition of transcription. Classical approaches investigate methylation patterns on a locus-by-locus basis. CpG islands associated with genes of interest are targeted for analysis. More recently, high-throughput methods have emerged to analyze the methylation state of a genome using microarrays of gene- or CpG island-specific oligonucleotides or PCR products. Here, we describe our approach, which yields a comprehensive methylation profile of a human chromosome at ~100 kb resolution, as it employs an array ~2100 clones (BACs, PACs, cosmids and fosmids) that represent a minimal tiling path covering the euchromatic portions of chromosome 1. We have found differences in methylation between different tissues from a normal human tissue panel. Interestingly, some hypomethylated clones contain genes that are tissue specific. We believe that this global view of methylation will aid in making gross comparisons among tissues or tumor samples, as well as point to specific CpG islands for more in-depth analysis. The approach can also be expanded to profile a variety of diseases associated with aberrant methylation. Identifying abnormal promoter methylation in diseases such as cancer would have a powerful impact on our understanding of the gene expression changes that contribute to the disease process.

2002

**Metaplasia of Hepatocytes into Pancreatic Acinar Cells During Mouse Liver Regeneration**

A. Tsou,[1] Y. Chau,[2] C. Liu,[1] C. Lin,[3] Y. Lu,[1] M. Hsu[4]; [1]Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei, Taiwan Republic of China, [2]Institute of Anatomy and Cell Biology, National Yang-Ming University, Taipei, Taiwan Republic of China, [3]Institute of Bioinformatics, National Yang-Ming University, Taipei, Taiwan Republic of China, [4]Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan Republic of China

Hepatocytes rarely divide but rapidly regenerate upon severe tissue losses by chemical or physical injuries. Numerous reports revealed observation of pancreatic cell phenotypes in the cultured hepatocytes, in animal livers and in patients with liver tumors embedded in a cirrhosis background. In addition, acute pancreatitis was known to occur in patients after liver transplantation. The molecular mechanism dictating the mutual interaction between adult liver and pancreas, however, is uncertain. In a global transcriptome study we detected activation of a cluster of 39 pancreatic exocrine genes during mouse liver regeneration induced by partial hepatectomy (PHx). Syn-expression of selected genes at early $G_1$ phase of liver regeneration was confirmed by qRT-PCR. Amylase from regenerating liver is *Amy2* by nucleotide sequence alignment. Amylase protein expression showed a portal-to-central vein gradient and peaked at early $G_1$ phase of liver regeneration as revealed by immunohistochemistry and western blot analysis. The observation that *Ptf1a* is specifically up-regulated suggests a possibility that hepatocytes may be induced to undergo metaplasia into pancreatic exocrine cells. Electron microscopic (EM) analysis of regenerating hepatocytes detected the granular structure similar in morphology to that of pancreatic zymogen granule and prominent rough ER structures characteristic of pancreatic acinus which were not found in control liver tissues. To examine whether pancreas is injured by the altered blood flow in hepatectomy, we examined the morphology of mouse pancreas after liver resection. EM analysis showed autophage structure and abnormal mitochondria in the pancreas during liver regeneration. The rough ER structure characteristic of pancreatic exocrine cells was also significantly decreased in the pancreas following hepatectomy. Liver-to-pancreas metaplasia during PHx-induced liver regeneration may constitute an early induction signal of liver regeneration and a signal to trigger recovery of pancreatic acinar cells from PHx-induced damages.

## Chromatin & Chromosomes II (2003-2020)

2003

**Comparative Proteome Analysis of Human Metaphase Chromosomes**

H. Takata, S. Uchiyama, N. Nakamura, S. Kobayashi, S. Matsunaga, K. Fukui; Biotechnology, Osaka University, Osaka, Japan

Chromatin was composed of DNA and many kinds of proteins and it takes a condensed structure called chromosome during mitosis. Although this chromosomal higher order structure is essential for cell division, protein components of chromosome and mechanism of chromosome condensation remain controversial. In this study, we aim to identify proteins which are essential for formation of the higher order structure by using comparative proteome analysis. Chromosomes were isolated from two different human cell lines, Ball-1 and HeLa S3, using polyamine procedure. Chromosomal proteins were extracted using an acetic acid method. Proteins were subjected to one-dimensional (1D) SDS-PAGE and two-dimensional electrophoresis (2DE). We use a radical free and highly reducing method (RFHR) for 2DE. RFHR method is useful to separate basic proteins, thus the method is more effective to detect chromosomal proteins that interact with DNA in chromosome. By CBB staining, about 200 spots were detected from the isolated chromosome protein with high reproducibility. Proteins were identified by mass spectrometry, MALDI-TOF MS or LC-MS/MS, and more than 100 proteins were identified from each cell line. Almost all chromosomal proteins were occupied by histone proteins and the amount of non-histone proteins is only 1/10 of histone proteins based on the information of quantitative analysis of identified proteins. By comparison of the amount between BALL-1 and HeLa S3, we identified some proteins whose amounts were conserved in both cell lines. These results suggest that some non-histone proteins are essential for chromosome higher order structure.

2004

**Molecular Analysis of Mitotic Chromosome Condensation Using a Quantitative Time-Resolved Fluoresence Microscopy Assay**

P. S. Maddox, N. Portier, A. Desai, K. Oegema; LICR, UCSD, La Jolla, CA

Chromosomes condense during mitotic entry to facilitate their accurate segregation. We have developed a quantitative method to monitor the kinetics of condensation in living cells by measuring progressive changes in the fluorescence intensity distribution of green fluorescent protein (GFP) fused to a core histone. In *C. elegans* and *Drosophila* embryos, condensation occurs during prophase in two temporally distinct steps that we term primary and secondary condensation. In *C. elegans*, where the histone variant CENP-A forms centromeric chromatin that runs along the entire length of each holocentric chromatid, both centromeric chromatin and condensin contribute to condensation. We show that preventing the assembly of centromeric chromatin results in a condensation defect that is kinetically distinct from condensin inhibition, and that the role of centromeric

chromatin in condensation is independent of its requirement for directing kinetochore assembly. Depletion of Aurora B, the mitotic histone H3 kinase reported to control condensin distribution on metaphase chromosomes, has no effect on condensation kinetics. In contrast to the SMC subunits of condensin, HCP-6, the CAP-D3 subunit of *C. elegans* condensin, requires CENP-A to localize to chromosomes, suggesting that it functions as a centromeric chromatin-specific adaptor for condensin. However, a quantitative comparison of HCP-6-depleted, SMC-4-depleted and CENP-A-depleted embryos indicates that HCP-6 and SMC-4 are of equal functional importance during condensation. This result suggests that the difference between the targeting of HCP-6 and SMC-4 to metaphase chromosomes reflects a change in chromosome architecture that occurs after prophase condensation.

2005

**Comparative Structural Biology of the Genome: Nano-scale Imaging Single Nucleus from Different Kingdoms Reveals the Chromatin Built Up on a 40 nm Structural Unit**

T. Kobori,[1] M. Kodama,[2] K. Hizume,[1] S. H. Yoshimura,[1] T. Ohtani,[3] K. Takeyasu[1]; [1]Graduate School of Biostudies, Kyoto University, Kyoto, Japan, [2]Kyoto University, Kyoto, Japan, [3]National Food Research Institute, Tsukuba, Japan

Eukaryotic genome is organized into the nucleus through several compaction steps. Core histones are well conserved among eukaryotes, form a 'beads-on-a-string' structure of nucleosomes together with DNA. Linker histones are also found in eukaryotes, and contribute to establish thicker 30-40 nm chromatin fibers. Interestingly, however, the linker histones do not always play a role in the genome packaging in some eukaryotes. Nevertheless, relationship between the genome structure and function seems to be generally conserved among eukaryotes. In this study, we comparatively analyzed the chromatin dynamics with different intrinsic properties; genome size, histone composition, and inter-nucleosomal distance. Namely, the nuclei isolated from human HeLa cells, chicken erythrocytes, and yeast *Schizosaccharomyces pombe* were structurally studied using a nanoscale imaging technique, atomic force microscopy (AFM). For this, we adopted the 'on-substrate' lysis procedure, which successively removes the nuclear membrane and the nucleoplasm while the chromatin and nuclear scaffold remain on the substrate, and, thus, allow us to look at the chromatin architectures in the nucleus at nano-scale. Our comparative structural analyses identified a common fundamental structural unit of the interphase chromosome. The high-salt treatment, which removed the nucleoplasm, caused a release of 40 nm fibers from the nuclei of all eukaryotic cells tested. The 80-100 nm bead structures were also observed on the surface of nuclei after the high-salt treatment. No structural population other than the 40 nm fiber and the 80 nm beads was clearly found, suggesting that the 40 nm fiber would fold directly up to the 80-100 nm beads regardless of eukaryotic species.

2006

**Older Human Cells Show More Complex Aneuploidy than Younger Cells after DNA Damage by Cobalt Chrome Particles**

D. Ladon, I. Papageorgiou, I. D. Learmonth, C. P. Case; Dpt. of Orthopaedic Surgery, University of Bristol, Bristol, United Kingdom

Humans are exposed to metals from the environment, industry and surgical implants. This study has explored whether cellular age influences the cytogenetic response to metal exposure (in this case cobalt-chrome alloy (CoCr) particles). Human primary fibroblasts at PD 5 (young) and PD35 (middle aged) were treated with CoCr particles (38.5 mg/ml) for 24 hours, harvested after 1, 2 and 15 days and karyotyped with M-FISH. 60 metaphases were analysed in each sample and in triplicate. Although the mitotic index (1000 cells/sample 3hr colchicine) of the resting young and older control cells was approximately the same (9-12%), there was a difference after short term metal exposure. The mitotic index was reduced in young cells to 2% at 1 and 2 days after exposure but was absent at this stage in older cells. By 15 days the mitotic index had partially recovered in both young and older cells (6%). The total incidence of structural and numerical chromosome aberrations was approximately the same after metal exposure of young and older cells (in metaphases that could be scored). There was little difference in the type of structural aberrations (deletions, fragments, translocations). However the type of aneuploidy was different. At day 15 the younger cells mostly showed loss of a chromosome (58% <46, 10% >46 5%>60) whilst older cells mostly showed a gain, often involving more than 60 chromosomes (7% <46, 47% >46, 29% >60). 30% of metaphases in older cells with more than 60 chromosomes showed evidence of a lack of cohesion and/or premature sister chromatid separation. This was only seen in 2% of metaphases in young cells. We conclude that cellular age in culture has a significant effect on the cytogenetic response to a mutagenic agent.

2007

**Fundamental Structure of Bacterial Chromosome Identified by Atomic Force Microscopy and the Reconstitution of Nucleosome-like-structure with Hu**

J. Kim,[1] K. Hizume,[1] S. Yoshimura,[1] R. Ohniwa,[1] C. Wada,[1] A. Ishihama,[2] K. Takeyasu[1]; [1]Department of Responses to Environmental Signals and Stresses, Graduate School of Biostudies, Kyoto University, Kyoto, Japan, [2]Division of Molecular Biology, Nippon Institute for Biological Science, Tokyo, Japan

In the *Escherichia coli* genome structure, one of the main structural proteins is Hu, which is known as histone-like protein. However, the molecular mechanisms of Hu for the formation of genome structure remain unclear, so it is not known weather there is beads-on-a-string structure like eukaryotic genome. In this study, we have tried to reconstitute the fundamental structure with 3 Kbp plasmid DNA (pBluescriptII) and Hu, and analyzed by AFM, to understand the mechanism of genome packing. As the reconstitution of eukaryotic nucleosome structures, salt-dialysis experiment was performed; DNA and proteins were mixed in the buffer containing 2 M NaCl and dialyzed against the buffer without NaCl. Dialysis was carried out until NaCl concentration became 50 mM, and subjected to AFM observation. As the results, a relaxed circular DNA which had a bead with a diameter 25 nm was observed, although the supercoiled plasmid was used for the reconstitution. Contour length was measured to be 930 nm, which is 70 nm (200 bp) shorter than 3 Kbp DNA. From the volume measurement of the volume of the beads and the length of DNA, 200 bp of DNA and 10 to 20 of Hu dimers seem to form this. These results suggest that the Hu could form the nucleosome like structure which has bead shape and resolve DNA supercoiling. When we just mixed DNA and Hu in low-salt buffer without dialysis process, beads structure was never observed. We had reported that 80-nm and 40-nm fiber structures were fundamental structure of the intact prokaryotic genome. From the combination of the observations of intact nucleoid and reconstituted Hu-DNA complex, we propose a structural model of the molecular mechanism of the fundamental structure of nucleoid.

2008

**Localization and Dynamics of Chromosomal Proteins in Human Cells**

D. No,[1] S. Matsunaga,[2] S. Uchiyama,[2] K. Fukui,[2] T. Azuma,[3] M. Nakagawa,[3] N. Ohmido[1]; [1]Graduate School of Cultural Studies and Human Science, Kobe University, Kobe, Japan, [2]Laboratory of Dynamic Cell Biology, Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Japan, [3]Division of Biosignaling, Research Institute for Biological Scienses, Tokyo University of Science, Noda, Japan

Chromosomes are essential for accurate segregation of replicated genomic DNA to daughter cells. It is not well-defined, which proteins contribute to the higher order structure and precise function of human chromosomes. To understand which proteins are important in chromosome structure and function, proteome analysis of isolated human chromosomes were carried out and listed 107 proteins could possibly relate to chromosome (Uchiyama, *et al.*, 2005). To identify the specific localization of these proteins on chromosome, we have generated polyclonal antibodies against candidate proteins and performed immunofluorescent staining using three types of chromosome samples of chromosomes in cells, hypotonic treated cells, and isolated chromosome from HeLa S3 cells. As the results of indirect immunofluorescent staining, FtsJ homolog 3 was localized to chromosome peripheral region on isolated chromosomes and spread chromosomes samples, FtsJ homolog 3 was localized at periphery of chromosomes and also on spindle body at metaphase and anaphase cells. These results show that FtsJ homolog 3 is a novel chromosomal protein. It is known that *ftsj* deletion results in a growth defect and altered ribosome profile in *E. coli* (Caldas, *et al*., 2000 and Bügl, *et al*., 2000). FtsJ is well-conserved as the heat shock protein from bacteria to human. Therefore, our data on FtsJ homolog 3 localizing on chromosomes and spindle body suggest that FtsJ homolog 3 might be also thought to be functional protein through cell cycle.

2009

**Mugsy, a *Drosophila* Nucleostemin-Like GTPase**

R. Rosby,[1] V. Robinson,[2] P. DiMario[1]; [1]Biological Sciences, Louisiana State University, Baton Rouge, LA, [2]Molecular and Cell Biology, University of Connecticut, Storrs, CT

Nucleostemin is a nucleolar GTPase implicated in stem cell and cancer cell proliferation (Tsai and McKay, 2002, 2005; Mistelli, 2005). We have identified a nucleostemin-like protein in *Drosophila* (CG3983 in cytological region 89E11 of chromosome 3R). The predicted protein that we call Mugsy is 581 amino acid residues in length. It displays 33.7% identity and 64.2% similarity to mammalian nucleostemin. Initial hydrolysis assays indicate the protein binds and hydrolyses GTP efficiently, despite its permuted GTPase motifs. The cDNA encoding the GFP-Mugsy was ligated into pUAST, a P element-containing transformation vector with yeast GAL4 upstream activation sequences. We prepared six homozygous transgenic lines that we crossed to the *daughterless-GAL4* driver line. Induced GFP-Mugsy localized primarily to nucleoli in all tissues examined. A small percentage also localized to the nucleoplasm. The interesting localizations were in larval salivary gland nuclei where GFP-Mugsy localized to the giant nucleoli as expected, but also to the condensed bands in the polytene chromosomes. This localization is similar to that of a modulo, another nucleolar protein known to localize to polytene bands, and to suppress position effect variegation (Perrin et al., 1998, 1999).

2010

**The Role of RNAi in Gene Silencing: Investigating VIG and Its Homologue CG11844**

K. D. Grim, S. C. R. Elgin, E. Gracheva; Lab of Dr. Sarah Elgin, Biology, Washington University in St. Louis, St. Louis, MO

We are studying the RNAi pathways in *D. melongaster*, in particular RNAi induced transcriptional silencing. Mutations in the RNAi genes *piwi, aubergine*, and *homeless*, have been shown to impact the formation of heterochromatin and the generation of histone H3 dimethylated at lysine 9 (H3K9me2) (Pal-Bhardra et al. 2004, Science 303, 669). A protein complex that has been implicated as playing a role in both RNAi-induced transcriptional and post-transcriptional silencing is the RISC complex (ribonuclease-containing RNA-induced silencing complex). A member of the RISC complex, VIG (Vasa Intronic Gene), is associated with heterochromatic regions of the polytene chromosome as shown by immunofluorescent staining (B. Brower-Toland, S. Elgin, unpublished data). However, mutations in VIG have no impact on a variegating *yellow* transgene. A search of the genome has identified a close homolog of VIG, putative gene CG11844, a locus with no characterized mutant alleles. Knock-down experiments in Drosophila Kc cells and VIG mutant flies demonstrate that the total H3K9me2 level does not change in a VIG deficient background; however when both VIG and CG11844 are knocked down in Kc cells, the H3K9me2 level drops. To evaluate the function of the CG11844 gene product in heterochromatin formation and thus transcriptional silencing *in vivo*, we are generating *D.melongaster* mutants deficient in CG11844 using 'Ends-In' targeting and deletion (method described in Xie and Golic, 2004, Genetics 168, 1477). A construct carrying sequences flanking the CG11844 gene, as well as a *hs-white* marker and the required restriction sites and FRT elements, has been prepared and will be injected into flies. The results should help us to further understand the mechanisms directing heterochromatin formation and maintenance. [Supported by NIH grant GM073190 to SCRE.]

2011

**Spatial Arrangement and Dynamics of Chromosomes in *S cerevisiae***

D. A. Bressan,[1] S. Jain,[1] E. Kim,[2] S. Haase,[2] J. Martin,[3] J. Kondev,[3] J. W. Sedat,[2] J. E. Haber[1]; [1]Rosenstiel Center and Department of Biology, Brandeis University, Waltham, MA, [2]Department of Biochemistry and Biophysics, University of California, San Francisco, CA, [3]Department of Physics, Brandeis University, Waltham, MA

The Recombination Enhancer(RE) is a cis-acting region located proximal to *HML* on the left arm of yeast chromosome III which controls donor preference during mating-type (*MAT*) switching. *MAT***a** preferentially recombines with *HML* on the left arm, whereas *MAT*alpha prefers *HMR* on the right arm. We marked several different chromosomal loci on chromosomes III and V using *lacO* and *tetO* arrays to which LacI- and TetR-fluorescent proteins bind. Using live-cell 3D deconvolution fluorescence microscopy, we found that the movement of *lacO* sequences adjacent to *HML* are strongly constrained in both *MAT*alpha and RE-deleted *MAT***a** strains, compared to *MAT***a** cells. In contrast, movement of *lacO* arrays adjacent to *HMR* or on the left arm of chromosome V is mating type-independent. *HML* is more strongly constrained in *MAT***a** re- and less constrained in *MAT***a** $RE^+$ compared with other sites. These data support models in which donor preference reflects the constitutive tethering of the left arm of chromosome III and that RE acts to relieve this tethering in *MAT***a** cells. Although the donor loci are not pre-paired with *MAT* prior to HO induction, we observed a mating type- and RE-dependent difference in the 3D configuration of *MAT*, *HML* and *HMR*. Kinetic analysis of synapsis between *MAT* and *HML* following double-strand break(DSB) induction by HO cleavage at *MAT* in wild-type and recombination-defective

mutants enables us to see the real-time dynamics of homology search that is the prelude to DSB repair. Recent imaging of the *MAT* and *HML* loci and the spindle pole body revealed both mating type- and locus-dependent differences in dynamic behavior of these regions. To study the rapid diffusion between different sites on chromosome III, we are analyzing images collected by a novel high-speed fluorescence deconvolution microscope developed by the laboratory of John Sedat.

2012

**Displacement of the Bromodomain Protein Brd4 from Chromosomes is Required for the Recovery from Anti-Microtubule Drug Induced Mitotic Arrest**

A. Nishiyama, K. Ozato; LMGR, NIH/NICHD, Bethesda, MD

Brd4 is a mammalian bromodomain protein that belongs to the conserved BET family. Brd4 associates with chromosomes during the entire course of mitosis through binding to acetylated histone H3 and H4. Mitotic cells are particularly susceptible to anti-microtubule drugs. To study the functional significance of Brd4-chromosome association, we examined the effect of anti-microtubule drugs. We found that Brd4 was displaced from mitotic chromosomes in response to all anti-microtubule agents tested, including the reversible agent, nocodazole. However, after nocodazole withdrawal, Brd4 was reloaded onto chromosomes, which coinciding with the resumption of mitotic progression. Several JNK inhibitors inhibited displacement of Brd4 from chromosomes induced by nocodazole. The JNK inhibitor also reversed Brd4 displacement when added following nocodazole, indicating that Brd4 displacement is a transient response and is controlled by JNK activation. We also found that cells treated with the JNK inhibitor were deficient in resuming mitosis after nocodazole removal, suggesting that Brd4 displacement has a functional role in restarting mitosis after drug withdrawal. Furthermore, we show that Brd4 heterozygous (Brd4+/-) cells are defective in reloading Brd4 onto chromosome after nocodaozole withdrawal, although Brd4 displacement by nocodazole was normal in Brd4+/- cells. Similar to JNK inhibitor treated cells, Brd4+/- cells were impaired in resuming mitotic progression after nocodazole removal, and showed increased chromosome missegregation and reduced cell division. Together, our study shows that Brd4 undergoes a dynamic movement from and to chromosomes after addition and removal of anti-microtubule agents. Brd4 dynamics triggered by these agents may represent a cellular response against drug induced mitotic stress, and may have a key role in facilitating the recovery from drug induced mitotic stress.

2013

**Linker Histone Dynamics, Structure, and Function in Interphase and Mitotic Sperm Chromosomes Assembled in Xenopus Egg Extracts**

B. S. Freedman, T. J. Maresca, R. Heald; Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Classic electron microscopic and nuclease digestion experiments have established that linker histones bind to nucleosomal arrays and compact them into a thicker chromatin fiber. It is unclear how these experiments, which were assayed at the scale of chromatin under processed conditions, relate to the *in vivo* organization of entire chromosomes. We recently addressed this issue using physiologically active *Xenopus* egg extracts and sperm nuclei that progress through the cell cycle. We found that replicated, mitotic chromosomes assembled in extracts depleted of the embryonic linker histone H1M (or B4) had a distorted morphology and were about twofold longer than normal (Maresca TJ *et al.*, 2005). Thus, H1M helps compact whole chromosomes, which is consistent with the classical role for linker histones at the smaller scale of nucleosomal chromatin. Currently, we are using this roughly physiological, vertebrate system to investigate several aspects of linker histone function: (1) To extend our findings from mitotic to interphase chromosomes, we are using fluorescence microscopy to measure nuclear size, chromosome morphology, and chromosome length (by FISH) in nuclei assembled with or without H1M. (2) H1M must first be loaded onto DNA during interphase in order to compact mitotic chromosomes. To understand the molecular mechanism underlying this loading step, we are therefore performing H1M immunoprecipitations to identify binding partners. We are also comparing the binding dynamics of H1M to chromosomes during interphase versus mitosis. (3) H1M has two major domains, a globular head and a highly basic, unstructured carboxyl tail. We are substituting these domains for the full-length protein to evaluate their separate contributions to chromosome morphology. We are also engineering phosphorylation-state H1M mutants, to investigate the long-standing mystery of why many linker histones are phosphorylated during mitosis.

2014

**A Novel Chromosome Compaction Step in Anaphase Revealed by Quantitative Fluorescence Microscopy in Living Cells**

F. Mora-Bermudez,[1] D. Gerlich,[1,2] J. Ellenberg[1]; [1]Gene Expression and Cell Biology/Biophysics, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, [2]presently at: Institute of Biochemistry, ETH, Zurich, Switzerland

Chromatin structure changes dramatically during mitosis, when chromosomes compact and congress in order to be correctly segregated to daughter cells. Despite its importance, this dynamic organization of chromatin *in vivo* remains poorly understood. To address mitotic chromatin compaction, we developed a set of quantitative assays based on fluorescence microscopy of intact mammalian cells stably expressing GFP-tagged core histones. First, we measure changes in the total volume occupied by fluorescent chromatin through mitosis, using high-resolution 4D confocal imaging. Second, pixel intensity statistics of fluorescent chromatin are analyzed. Both assays show that the majority of mitotic compaction occurs during prophase. Surprisingly, after sister chromatid segregation, in late anaphase, chromosomes reach a compaction level even higher than in metaphase. To characterize this unexpected late compaction step, we developed a photoactivation assay to label confined regions in single chromosomes. Quantitative analysis shows that, at the time of the anaphase recompaction, protruding chromosome arms shorten by further compaction toward the rest of the chromatin mass, rather than by relocation within the anaphase. To identify the forces underlying this event, we tested whether microtubules are required. In untreated cells, compaction of all chromosomes was completed within 12 minutes after segregation. In cells were microtubules were acutely depolymerized by nocodazole immediately after segregation, 83% of measured chromosomes failed to fully compact with normal timing. This suggests that anaphase microtubules not only segregate and direct chromosomes to daughter cells, but may also participate in their recompaction, to form a single mass of chromatin before nuclear envelope reassembly. We are currently further characterizing this novel compaction event and are probing the possible involvement of chromatin-intrinsic factors.

2015

**Ubiquitination at DNA Breaks**

L. Postow, H. Funabiki; The Rockefeller University, New York, NY

Double strand DNA breaks induce two separate pathways: the DNA damage checkpoint response and double strand break repair. In mammalian cells, the most common repair mechanism is nonhomologous end joining (NHEJ). Both the checkpoint response and NHEJ can be reconstituted in extracts made from the eggs of the frog *Xenopus laevis.* Using *Xenopus* egg extract, we developed a system to biochemically identify proteins bound to damaged DNA structures. Such proteins are likely involved in either the checkpoint response or repair. Biotinylated DNA was conjugated to magnetic streptavidin-coated beads, and proteins binding these DNA-coated beads were analyzed. Many of the proteins that bound to these DNAs were well-known damage response proteins, including Ku70, Ku80, the DNA-PK catalytic subunit, Werner's helicase, and RPA. By mass spectrometry, we also found ubiquitin and several subunits of the 26S proteasome. In addition, the majority of the polyubiquitin conjugated to these DNA-bound proteins has the K48 branch point characteristic of proteins targeted to the proteasome. These results suggest that some proteins bound to the damaged DNA are ubiquitinated in higher eukaryotes, and moreover that some of these ubiquitinated proteins are subsequently degraded by the proteasome. We are investigating which damage-bound proteins are ubiquitinated, and the role of this ubiquitination in the damage response.

2016

**Cak1p/CDK Interactions with Ctf7p-dependent Sister Chromatid Cohesion**

A. Brands, R. V. Skibbens; Biological Sciences, Lehigh University, Bethlehem, PA

High fidelity chromosome segregation requires that sister chromatids become paired or tethered together during S-phase. An essential step in chromatid pairing, termed cohesion establishment, involves Ctf7p/Eco1p (herein Ctf7p)(Skibbens et al., 1999; Toth et al., 1999). At present, there is no molecular mechanism known that coordinates cohesion establishment to S-phase. To understand how Ctf7p establishment activities are regulated, we performed a genome-wide synthetic lethal screen using the conditional *ctf7-203* allele to identify mutations in essential factors not present in most micro-array and high-throughput screens. To date, we identified 10 non-sectoring strains - representing new candidates for cohesion establishment factors. We cloned the first of these as a novel *cak1* allele. Cak1p is a serine/threonine kinase that phosphorylates Cdc28p - the major Cyclin-Dependent Kinase or CDK in budding yeast (Espinoza et al., 1996; Kaldis et al., 1996). Importantly, Cak1p is required for timely S-phase progression, potentially coordinating Ctf7p function to Cak1p/Cdc28p activation. Similar to the temperature sensitivity of *cak1* alleles (Cross and Levine, 1998), we find that the synthetic lethality of *cak1 ctf7* double mutants is bypassed by *cdc28* hypermorphs. These data suggest that cohesion establishment is tightly regulated by CDK activity.

2017

**Construction of the Cytogenetic Map of Maize Chromosome 9 by FISH and Integration of Pachytene Chromosome Structure and Recombination Frequency**

C. Wang, L. Harper, W. Cande; Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA

Meiotic recombination takes place during early prophase I of meiosis and genetic maps are based on the percentage of recombination between markers. However, recombination events are is not evenly distributed throughout the genome. To correlate the gene positions and chromosome structure with recombination frequency and linkage maps, we developed a novel high-resolution single copy FISH technique in maize to map genes on pachytene chromosomes for construction of a cytogenetic map. Since pachytene is the stage in which recombination is completed and most recombination occurs within genes, placement of genetically mapped gene on pachytene chromosomes by FISH will not only integrate chromosome structure and the genetic map, but also provide biological information about the global positions of genes, distribution of recombination events, and may shed light on the process of meiotic homologous pairing. We optimized our FISH procedure and successfully mapped nine single-copy genes on chromosome 9 that had been previously genetically mapped. All probes were directly labeled with fluorophores, and the smallest probe used was 3.1 kb. Two loci, *bz1* and *tac7077*, which are less than 1 cM apart, can be distinguished as two distinct signals. The centromere region 0.3 cM on the genetic map spans ~30% of short arm of chromosome 9; however, the distal region of 9S that represents almost 36 cM on the genetic map is only 25% of short arm. We found that the dramatic reduction of recombination between markers around the centromere corresponds to the pericentric heterochromatic region.

2018

**The Nuclear Envelope and Sister Chromatid Cohesion Factors Play a Direct Role in Telomere Length Homeostasis**

L. M. Antoniacci, R. V. Skibbens; Biological Sciences, Lehigh University, Bethlehem, PA

The nuclear envelope plays key regulatory mechanisms in both the spatial organization of chromatin within the nucleus and gene transcription. Mps3p/Nep98p (herein termed Mps3p) is an integral nuclear envelope protein with a single transmembrane domain that is recruited to the spindle pole body half bridge. Previous studies reported that Mps3p is essential for spindle pole body duplication (Jaspersen et al., 2002; Nishikawa et al., 2003). More recently, Mps3p was shown to perform a critical function in sister chromatid cohesion (Antoniacci et al., 2004). Here, we provide new evidence for a novel regulatory mechanism in which a telomerase-assembly component, Est1p, associates with Mps3p - suggesting that Est1p is sequestered to the nuclear envelope. We also report that Ctf7p/Eco1p (herein termed Ctf7p), previously found to interact with Mps3p, also physically and genetically interacts with Est1p and functions in telomere length regulation. Importantly, *mps3* and *ctf7* mutant cells exhibit similar telomere length increase defects - opposite that of telomere length decrease defects reported for *est1* mutant cells. These findings reveal a nuclear envelope-based balancing act in which associated factors promote telomere growth and shortening - altering telomere homeostasis.

2019

**Bimodal Dynamic Behavior of the Origin Regions of the Two *Vibrio cholerae* Chromosomes**

A. Fiebig, K. Keren, J. A. Theriot; Biochemistry, Stanford University, Stanford, CA

Chromosome partitioning is an essential process for all cells. Eukaryotic cells use the mitotic apparatus and regulatory checkpoints to faithfully segregate their chromosomes. Less is known about how bacteria accomplish the same task. Although most bacterial genomes are contained on a single circular chromosome, numerous bacterial species encode essential genes on more than one chromosome. These studies focus on *Vibrio cholerae*, a human pathogen whose genome is divided between two circular chromosomes. We used fluorescent repressor-operator systems to visualize the origin region of both chromosomes in live cells. Time-lapse analysis revealed important similarities and differences in the behavior of the two origins ($ori_I$ and $ori_{II}$). Both exhibit two modes of behavior we term "at home" and "traveling" that are correlated with progress through the

cell cycle. When at home, both origins move randomly within an ellipsoidal caging domain and their movements are comparable over 20-1500 second time scales. The home positions, however, are distinct. Home for $ori_I$ is near but not at the pole, while $ori_{II}$ is near the mid-cell and after segregation near the quarter-cell. Furthermore, each origin employs unique mechanisms to maintain the home position. The positioning mechanism for $ori_I$ measures absolute distance while that for $ori_{II}$ measures relative distance from the pole. During segregation, origins travel to find and establish a new home. $ori_I$ segregates asymmetrically from the old pole and $ori_{II}$ segregates symmetrically from the mid-cell. In both cases movement is directed, speed is comparable, and cell growth accounts for about half of the motion observed. Thus while the home positions, mechanisms to maintain the home position, and pattern of segregation differ, the local dynamic behavior of the origins of the two *V. cholerae* chromosomes are similar both at home and while traveling.

2020

**Telomere Shortening in Skeletal Muscle from MDX Mice**

T. C. Lund,[1] S. A. Nelson,[2] R. W. Grange,[3] D. A. Lowe[2]; [1]Department of Medicine, University of Minnesota, Minneapolis, MN, [2]Department Physical Therapy and Rehabilitation, University of Minnesota, Minneapolis, MN, [3]Dept. of Human Nutrition, Foods and Exercise, Virginia Polytechnic Institute and State University, Blacksburg, VA

Telomeres are the DNA sequences at the ends of chromosomes that keep tally of the number of cell divisions. Prior studies have shown that when telomeres become too short, chromosomes break and cell senescence or apoptosis occurs. Muscular dystrophy (MD) is a common X-linked neuromuscular disease in which the lack of the protein, dystrophin, causes muscle fiber damage. While previous studies have shown satellite cells are maintained in dystrophic muscle, they are reduced in number and this likely contributes to the pathogenesis of MD . We hypothesize that this is in part due to significant telomere shortening in these cells. Furthermore, our study stands out as the first to evaluate the diaphragm muscle for telomere dynamics. DNA was isolated from diaphragm and tibialis anterior muscles from mdx and wildtype mice aged 20, 40, 80, 100, 240, and 600 d (n=2-6 per age). Telomere length was determined by Southern blot. We found that telomere length was not different between muscles from 20, 40 or 80 d mdx and wildtype mice ($P>0.05$). Telomere length in diaphragm muscles from 100 and 600 d mdx mice was 84 +/- 3.8% and 59 +/- 1.4% of that from age-matched wildtype mice ($P<0.05$). In tibialis anterior muscles, telomere shortening was not evident in 100 d mdx mice (103 +/- 3.4% of wildtype) but in 600 d mice it was 68 +/- 2.3% of wildtype ($P<0.05$). These findings indicate that muscle satellite cells undergo telomere erosion and conceivably have a more finite life span in vivo than previously appreciated. Although considered to have stem cell characteristics, satellite cells in mdx muscle are perhaps under so much pressure to divide that they succumb to telomere erosion limiting their lifespan and their ability to repair damage muscle.

## Mammalian Development (2021-2046)

2021

**Embryonic Genome Activation in Porcine**

H. Odaman-Mercan,[1] M. Misirlioglu,[1] H. Sagirkaya,[2] M. Crenshaw,[1] E. Memili[1]; [1]Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS, [2]Veterinary Faculty, Uludag University, Bursa, Turkey

The embryonic genome activation (EGA) is the most critical event of early mammalian embryogenesis. Still not well-understood in the porcine, EGA is remarkable with regard to the number of embryonic genes that must be activated, maternal messages that must be degraded, and the dramatic reprogramming of embryonic gene expression setting the stage for events associated with first differentiation and implantation. The objective of this study was to define timing of transition from maternal to embryonic control of development, and transcriptional patterns of two developmentally important genes, Glut1 and Hsp70, in preimplantation porcine embryos. In three groups, in vitro fertilized porcine oocytes were cultured in NCSU23 medium supplemented with 0.4% BSA at 39ºC, 5% $CO_2$. Presumptive zygotes (Group I) and 2- to 4-cell embryos (Group II) were cultured with specific inhibitor of mRNA synthesis α-amanitin (50 µg/ml) between 5 and 36 hours post insemination (hpi) and 36 and 48 hpi, respectively. Following α-amanitin exposure, the embryos were washed three times with TL-HEPES and then transferred into the NCSU23 medium without α-amanitin. Embryos in the Control Group were cultured without α-amanitin. While the control embryos reached to the blastocyst stage, α-amanitin treated embryos both in Groups I and II were arrested at the 8- to 16-cell stage. Glut1 and Hsp70 gene expressions, in the three groups were determined by quantitative RT-PCR relative to the transcripts of housekeeping gene β-Actin. In Group I, Glut1 and Hsp70 gene expressions were not different from that of Control Group. However, Hsp70 gene in Group II was significantly down-regulated as compared to Control Group ($p<0.001$). These results indicate that embryonic gene expression is essential for cell cleavage beyond 8- to 16-cell stage, and Glut1 and Hsp70 are maternal until 2-cell stage with Hsp70 being both maternal and embryonic at the 4-cell stage.

2022

**Reset of the *Saccharomyces cerevisiae* Aging Clock by Meiosis during Prophase Recombination Period**

V. Lukose,[1] S. DeSantiago,[2] L. Cahoon,[1] M. Wu,[1] K. Lancaster,[1] B. Rockmill,[3] L. L. Mays Hoopes[1]; [1]Biology and Molecular Biology, Pomona College, Claremont, CA, [2]Molecular Biology, Pomona College, Claremont, CA, [3]Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT

Asymmetric replicative aging occurs during budding or mitosis in *Saccharomyces cerevisiae.* The bud is reset to 0 g (generations) while the mother cell gains 1 g per mitosis. Meiosis was not previously known to reset yeast aging. We found that meiosis resets the aging clock. **Methods:** We magnetically sorted 8 g BR19191-8B wild type cells using Pierce EZ-Link Sulfo NHS LC biotin and Miltenyi anti-biotin beads, and induced meiosis on potassium acetate sporulation medium. **Results:** The cells germinating from these ascospores had life spans nearly as long as young cells, much longer than 8 g cells. All four spores were reset. We next examined resetting in meiotic mutants. Ndt80 initiates a transcription wave at pachytene (prophase I), after most recombination/repair. Mutant *ndt80* cells stall at pachytene but they can be 'returned to growth' by restoring rich medium. The 8 g *ndt80* cells 'returned to growth' are already reset; therefore, meiotic resetting occurs before pachytene. We also examined *spo11* homozygous diploids, which cannot form the first double strand break (DSB) required to initiate recombination. We found that *spo11* does not reset its aging clock during meiosis. **Conclusion:** We conclude that resetting occurs between the time of DSB formation and Ndt80 expression during prophase I. We hypothesize that DSB formation and DNA recombination may be involved in the resetting.

2023

**Increased Protein Arginine Methylation during the Myoblast Fusion**

S. Kim,[1,2,3] S. Kim,[2] C. Uhm[1,2,3]; [1]Anatomy, Korea university, Seoul , Korea, Republic of Korea, [2]Brain Korea 21 Biomedical Sciences, Seoul, Republic of Korea, [3]Korea Lung Tissue Bank, Seoul, Republic of Korea

Skeletal muscle is developed from multinucleated myocytes formed by the fusion of mononucleated myoblasts. The fusion of myoblasts is associated with many changes in muscle biology such as cessation of DNA synthesis, production of contractile proteins, and marked alterations in enzymatic activity. Before fusion, myoblasts irreversibly withdraw from the cell cycle. This cell cycle exit is required for the normal progression of fusion, and the development of the contractile machinery. Recent report [Chen et al., JBC 277:4324, 2002] suggests that protein arginine methyltransferase (PRMT) 4 is involved in the chromatin remodeling and gene expression related to the muscle differentiation in C2C12 cells, suggesting a possible role as one of the controlling mechanisms of the cell cycle exit of myoblasts. In this study the expression of PRMT1 and PRMT5 during the myoblast fusion in primary muscle culture was investigated. Rat myoblasts isolated from E21 SD rats were cultured as reported previously [Daniels et al., Micr Res Tech 49:26, 2000]. Spontaneous myoblast fusion was blocked by adding EGTA into the culture media, and the fusion burst was induced by changing with normal media. Proteins were extracted at the stages of prefusion, fusion arrest, fusion burst, and postfusion. The extracted proteins were incubated with *S*-adenosyl-L-[*methyl*-$^{3}$H]methionine, and subjected to SDS-PAGE followed by fluorography. Western immunoblot using antibodies against PRMT1 and 5 were performed. Expressions of both PRMT1 and PRMT5 were increased during the fusion burst, which decreased to the prefusion level after fusion. Western immunoblot data was similar to the fluorographic results. The present study suggests that PRMT1 and PRMT5 may also play an important role in the myoblast fusion similar to PRMT4. Especially PRMT1 and *S*-adenosyl-L-[*methyl*-$^{3}$H]methionine(AdoMet)-dependent methylation are necessary for the biochemical and morphological changes of myoblasts during the fusion stage.

2024

**Mild Heat Shock Induces Differentiation of Mammalian and Avian Myoblasts**

T. Yamaguchi, Y. Atomi; University of Tokyo, Art and Science, Atomi's Laboratory, Tokyo, Japan

Skeletal myogenesis is a well-orchestrated cascade of events regulated by multiple signaling pathways. Heat shock response is one of the intracellular defense mechanisms against stressful conditions, which leads to the preferential transcription and translation of heat shock proteins (Hsps). In this study, we investigated the direct effects of temperature on differentiation of cultured C2C12 myoblasts. Compared with the control cultures at 37°C, incubation at 39°C increased myotube diameter. Although the myotube formation at 41°C appeared earlier, their diameters were reduced. The levels of the Hsps (Hsp70 and alphaB-crystallin) were up-regulated with increased temperature. Muscle specific transcription factors, MyoD and myogenin, and extracellular signal rgulated protein kinase (ERK) were induced above at 39°C culture, relative to the 37°C. Moreover, inhibitition of mitogen-activated protein kinase/ERK kinase (MEK) activation by PD98059 suppressed myotube formation. These data suggest that treatment with a mild heat shock might induce C2C12 myotube enlargement via the MEK/ERK pathway. The similarity of the response to mild heat shock was observed in chick and human myoblasts in primary culture of skeletal muscle.The delicate balance of signaling pathway due to a slight change of temperature might be involved in myotube enlargement.

2025

**Axial Elongation in Mouse Embryos Involves Mediolateral Cell Intercalation Behavior in the Paraxial Mesoderm**

W. Yen,[1] C. Burdsal,[2] A. Periassamy,[3] A. E. Sutherland[1]; [1]Cell Biology, University of Virginia, Charlottesville, VA, [2]Biology, Tulane University, New Orleans, LA, [3]Biology, University of Virginia, Charlottesville, VA

The cell mechanical and signaling pathways involved in gastrulation have been studied extensively in invertebrates and amphibians, such as *Xenopus*, and more recently in non-amniote vertebrates such as zebrafish and chick. However, because culturing mouse embryos extra-utero is very difficult, this fundamental process has been least characterized in the mouse. As the primary mammalian model for genetics, biochemistry, and the study of human disease and birth defects, it is important to investigate how gastrulation proceeds in murine embryos. We have developed a method of using 4D multiphoton excitation microscopy and extra-utero culture to visualize and characterize the morphogenetic movements in mouse embryos dissected at 8.5 days of gestation. Cells are labeled by expression of an X chromosome-linked EGFP transgene. This method has provided a unique approach, where, for the first time, patterns of cell behavior in the notochord and surrounding paraxial mesoderm can be visualized and traced quantitatively. Our observations of mouse embryos reveal distinct differences in patterned cell motility relative to other vertebrate models such as *Xenopus*, where axial extension is driven primarily by mediolateral oriented cell behaviors in the notochord and paraxial somatic mesoderm. Unlike *Xenopus*, cells within the mouse notochord are not aligned, although they are elongate. In contrast, cells within the paraxial mesoderm are both elongated and aligned perpendicular to the midline. In addition, these cells are observed to translocate towards the midline, consistent with a role for paraxial mesoderm in driving convergence and extension. These cell behaviors are similar to those characterized in the axial mesoderm of frog embryos during convergence and extension, and suggests that tissues may play different roles in axial elongation between the frog and the mouse.

2026

**Potential Role of Pinin in Regulation of Wnt Signaling and Neural Crest Cell Migration during Mouse Development**

J. Joo,[1] Y. Lee,[2] M. R. Jackson,[1] P. Oh,[2] S. P. Sugrue[1]; [1]Anatomy and Cell Biology, University of Florida, Gainesville, FL, [2]Physiology and Functional Genomics, University of Florida, Gainesville, FL

Numerous studies have demonstrated that Pinin (Pnn), a nuclear and cell adhesion-related protein, functions as a critical mediator of various multiprotein complexes involved in regulation of gene expression, pre-mRNA splicing, and cell adhesion. To investigate biological role of Pnn, we generated conditional Pnn knockout mice with three *loxP* sites (3f) flanking exon 3 to exon 8 of *Pnn* as well as a neomycin resistance cassette. EIIa-Cre mice were utilized to generate constitutive *Pnn*-null (1f) and *Pnn*-conditional (2f) alleles. The *Pnn*$^{1f/1f}$ embryos exhibited lethality at peri-implantation stages. Interestingly, *Pnn*$^{3f}$ appeared to be a hypomorphic allele, which resulted in reduced Pinin protein expression due to incorrect splicing. The *Pnn*$^{3f/3f}$ mice die around birth and exhibit pleiotropic phenotypes, including disorganized epidermis, subepidermal blisters, cleft palate, axial skeletal defects, cardiac outflow tract defects, and anterior eye segment dysgenesis. Since most of these phenotypes are related to

defective neural crest cells (NCC) of which Wnt signaling is critical for the proliferation and migration, we investigated whether Wnt activities are affected in *Pnn*$^{3f/3f}$ ES cells. We found that the activity of Wnt downstream transcription factors, TCF/LEF, is greatly upregulated in *Pnn*$^{3f/3f}$ ES cells. Furthermore, the TCF/LEF activity was also observed to be altered in *Pnn*$^{3f/3f}$ mice. Finally, NCC-specific ablation of *Pnn* by Wnt1-Cre mice revealed severe cardiac and craniofacial defects similar to those of DiGeorge Syndrome, suggesting an essential role of Pnn in the proliferation and/or migration of NCCs. In summary, the results suggest that Pnn is involved in the regulation of Wnt signaling pathway and NCC migration.
Supported by: NIH Grant EY07883

2027

**Isolation, Characterization and Secretion of *Xenopus laevis* Embryonic Epidermal Lectin**

S. Nagata,[1,2] N. Fujita[2]; [1]Animal Science, University of California at Davis, Davis, CA, [2]Chemical and Biological Sciences, Japan Women's University, Tokyo, Japan

The *Xenopus laevis* embryonic epidermal lectin (XEEL) is a novel member of a chordate lectin family including mammalian intelectins, frog oocyte cortical granule lectins and plasma lectins in lower vertebrates and ascidians. To elucidate roles of XEEL in embryonic development, we isolated XEEL from tailbud embryos by affinity chromatography on a galactose-Sepharose column. The XEEL protein is a homohexamer of 43-kDa N-glycosylated peptide subunits, requires $Ca^{2+}$ for saccharide binding and shows a higher affinity to pentoses than hexoses and disaccharides. The recombinant XEEL (rXEEL) produced by transfected HEK-293T cells is similar to the purified XEEL in its molecular nature and saccharide-binding properties. Substitution of Asn-192 to Gln removed the N-linked carbohydrate and inhibited secretion of rXEEL, but did not abolish the activity to bind to galactose-Sepharose. Examination of chimeric proteins between rXEEL and rXCGL suggests that the C-terminal segment of XEEL is also important for secretion. The XEEL content of embryo, as estimated by Western blot analyses, increases during neurula/tailbud stages and declines after one week post-fertilization. Immunofluorescence and immuno-electron microscopic analyses showed localization of the XEEL protein in a typical secretory granule pathway of non-ciliated epidermal cells. When tailbud embryos were cultured in the standard medium, XEEL was accumulated in the medium, indicating secretion of XEEL into the environmental water. The rate of XEEL secretion greatly increased at around the hatching stage and stayed at a high level during the first week after hatching. Mammalian intelectins with similar molecular nature and carbohydrate specificity have been implicated in anti-microbial innate immunity in intestinal mucosa. XEEL may have a similar role to protect hatched embryos and early larvae against pathogenic microorganisms in the environmental water.

2028

**Ena/vasp Proteins are Required for Cortical Organization, Neuritogenesis and Axonal Outgrowth During Vertebrate Development**

A. V. Kwiatkowski,[1] D. A. Rubinson,[1] E. W. Dent,[1] J. D. Leslie,[2] F. B. Gertler[1]; [1]Biology, Massachusetts Institute of Technology, Cambridge, MA, [2]Cancer Center Research UK, London, United Kingdom

Ena/VASP proteins regulate actin dynamics, and previous studies have implicated Ena/VASP proteins in neuronal migration and axon guidance. To investigate the requirement of Ena/VASP proteins in nervous system development, we disrupted the remaining Ena/VASP protein, EVL, in the mouse and crossed it to existing Mena (Lanier et al., 1999) and VASP (Aszodi et al., 1999) mutant lines. Within the peripheral nervous system, deletion of all three Ena/VASP proteins resulted in poorly formed trigeminal and spinal nerves that exhibited reduced outgrowth and guidance defects. Histological analysis of the cortex revealed a disorganized intermediate zone with poorly formed or absent fiber tracts, suggesting a defect in axonal formation or extension from cortical neurons. Strikingly, Ena/VASP-deficient brains exhibited a severe form of cobblestone (type II) lissencephaly, in which cortical neurons migrated beyond the marginal zone and established ectopias within the subarachnoid space. The severity of these ectopias reflect possible defects in pial membrane formation and neuronal migration. Surprisingly, in contrast to neurons in the cortical plate, axonal outgrowth was observed from ectopic neurons, suggesting that factors present outside the cortex, possibly in the pial membrane, induce outgrowth in the absence of Ena/VASP. When cultured in vitro, cortical neurons from Ena/VASP-deficient brains fail to undergo neuritogenesis (see accompanying abstract by Dent et al.), consistent with outgrowth defects observed in vivo. However, this defect was rescued when neurons were plated onto laminin, the predominant matrix protein of the pial membrane that is absent from the cortical plate. These results suggest Ena/VASP proteins and laminin function in independent pathways to promote neurite initiation. This study defines new roles for Ena/VASP proteins in neuritogenesis, axonal outgrowth, and cortical organization during vertebrate nervous system development.

2029

**Tbx1 is Required for Epithelial-mesenchymal Interactions Necessary for Inner Ear Development**

E. M. Braunstein,[1] J. S. Arnold,[1] T. Ohyama,[2] A. Groves,[2] E. B. Crenshaw,[3] J. Adams,[4] B. Morrow[1]; [1]Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY, [2]House Ear Institute, Los Angeles, CA, [3]Childrens' Hospital of Philadelphia, Philadelphia, PA, [4]Massachusetts Eye and Ear Infirmary, Boston, MA

Epithelial-mesenchymal interactions between cells of the otic vesicle and periotic mesenchyme are necessary for normal patterning of the inner ear. The T-box transcription factor, *Tbx1*, is required for proper ear development and is expressed both in the otic vesicle and surrounding periotic mesenchyme (POM) during embryogenesis. To determine the role of *Tbx1* in each tissue, we have generated a conditional allele of this gene. Pax2-Cre was used to drive otic vesicle specific ablation of *Tbx1*, while Brn4-Cre was used to ablate *Tbx1* in the POM. Inactivation of *Tbx1* in the otic vesicle resulted in hypoplasia of the inner ear sensory structures and duplication of the cochleovestibular ganglion, similar to *Tbx1* null mice. Ablation of *Tbx1* in the POM resulted in severe structural malformations of the both otic capsule and cochlea. Because the endogenous *Brn4* gene is also inactivated in this conditional mutant, we are currently comparing our phenotype to that of the Brn4 null mouse. These data suggest that *Tbx1* plays a role in signaling between the epithelial and mesenchymal cells responsible for proper patterning of the inner ear. Based on a yeast-2-hybrid screen for binding partners of Tbx1, we hypothesize that Tbx1 may mediate reciprocal signaling between these cells via interaction with proteins localized to focal adhesions and the actin cytoskeleton.

2030

**Immunocytochemical Distribution of Somatotrophs, Mammotrophs, and Mammosomatotrophs in Porcine Anterior Pituitary**

J. Lee,[1] M. H. Stromer,[2] S. Jeftinija,[3] K. Jeftinija,[3] C. G. Scanes,[4] L. L. Anderson[1,3]; [1]Department of Animal Science, Iowa State University, Ames,

IA, [2]Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA, [3]Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA, [4]Department of Poultry Science, Mississippi State University, Starkville, MS

The existence of a novel anterior pituitary cell type, the mammosomatotroph (MS), secreting both growth hormone (GH) and prolactin (PRL) was proposed to function as transitional cells or progenitor cells between somatotrophs (GH cells) and mammotrophs (PRL cells). Age-associated changes were found in the spatial distribution patterns of GH cells both radially and at different axial levels in the porcine pituitary. Fluorescence immunocytochemistry was used to identify GH, PRL, and MS cells and compare their distribution patterns in anterior pituitary glands from newborn and prepubertal stages of pigs. Anterior lobes were sampled at 3 positions in each of 5 radial regions in each of 3 levels perpendicular to the gland axis. While GH cells were densely populated in regions 1, 3, and 5, PRL cells had a high number and immunointensity of fluorescence along all regions close to the intermediate lobe. There were gradually increasing numbers of GH and PRL cells in region 3 from proximal to distal levels at all ages. Double immunofluorescence positive MS cells were distributed in regions 1, 3, and 5 near the intermediate lobe at the proximal level. Furthermore, there was a high population of Connexin 43, a known gap junctional protein, in region 3 at the all levels of depth. Differential immunoreactivity of GH, PRL, and MS cells across regions and ages may reflect heterogeneity or transitional status of these endocrine cells. From these results, we suggest that there may be regional specificity of cellular differentiation and transformation involving cell-to-cell communication through gap junctions to facilitate GH and PRL secretion in the need for endocrine regulation during the rapid growing period in the young pig. Supported by research grant USDA/CSREES NRI 2003-35206-12817 (L.L.A., S.J., and C.G.S).

2031

**Lineage Analysis of the Atrioventricular Myocardium during Cardiac Valve Development**

V. Gaussin,[1] L. Emile,[1] J. B. E. Burch[2]; [1]Cell Biology and Molecular Medicine, UMDNJ-New Jersey Medical School, Newark, NJ, [2]Fox Chase Cancer Center, Philadelphia, PA

The formation of the atrioventricular (AV) valves is a complex event, reflected by the high incidence of congenital valvular defects. Several lineage studies in the mouse indicated that the AV leaflets derive mostly from the mesenchymal cushions in the AV canal. These cushions are in contact with AV myocardium during development suggesting a role for this myocardium during valvulogenesis. Here, we analyzed the contribution of AV myocardium to the formation of the AV valves. For this, we used transgenic mice expressing Cre under the control of a cGATA6 heart-specific enhancer and LacZ-reporter mice for Cre activity (R26R). In E11.5 cGATA6-Cre/R26R mice, Cre-mediated DNA recombination was largely confined to the myocardium in the AV canal and was more robust on the right and posterior sides. At E14.5, LacZ-positive cardiac myocytes were found in the myocardial layer present in the tricuspid mural and posterior leaflets, and in the myocardium in contact with the septal leaflet of the mitral valve. They were also identified in the myocardium at the atrial border of the developing annulus fibrosus. One week postnatal and in adult hearts, LacZ-positive cells contributed to the tricuspid mural and posterior leaflets, the mitral septal leaflet, and the atrial border of the annulus fibrosus. We previously reported that when Alk3, the type Ia receptor for bone morphogenetic proteins, was deleted in these cells, defects were seen in the same leaflets, i.e. the tricuspid mural leaflet and mitral septal leaflet were longer, the tricuspid posterior leaflet was displaced and adherent to the ventricular wall, and the annulus fibrosus was disrupted resulting in ventricular preexcitation. In conclusion, AV myocardium is essential for AV canal development, and contributes to the morphogenesis of AV valves and annulus fibrosus.

2032

**Identification of Candidate Genes in a Transgenic Mouse Model of Urogenital Development and Obstruction**

K. M. McHugh, S. Singh, M. Chilton, A. Bailes, D. Pitre, B. Coley, H. Auer, K. Kornacker, C. M. Bates, M. L. Robinson; Pediatrics, Columbus Children's Research Institute, Columbus, OH

Diseases of the urinary bladder affect an estimated 35 million people in the United States, with end-stage renal failure in children alone estimated to cost over 15 billion dollars annually. Despite the prevalence of bladder disease, little is currently known regarding the mechanisms controlling these processes. We have identified a unique insertional mutant mouse model, designated *mgb,* that develops *in utero* megabladder resulting in variable hydroureteronephrosis and chronic renal failure secondary to obstructive uropathy. Homozygous *mgb* mice possess a primary defect in bladder smooth muscle development that is apparent by embryonic day 15, and whose functional outcome appears gender-specific. Interestingly, heterozygous *mgb* mice possess an intermediate phenotype that appears to result in altered bladder function. FISH analysis indicates that the *mgb* phenotype is the result of a translocation of a defined segment of chromosome 16 into chromosome 11. Using a unique microarray approach, we have identified a subset of genes from the translocated piece of chromosome 16 that are consistently over-expressed in *mgb* mice. This approach has permitted the rapid identification of two primary candidate genes, and represents a potentially exciting technique for the identification of target genes involved in insertional mutagenesis. In conclusion, we have utilized a novel microarray-based approach to rapidly identify the potential candidate gene(s) involved in the production of the *mgb* phenotype. *Mgb* mice represent an excellent model for the study of normal and pathogenic bladder development, including the postnatal progression of chronic renal failure resulting from obstructive uropathy.

2033

**Regulation of Pocket Protein Expression by Activating Transcription Factor-2 (ATF-2)**

D. S. Vale-Cruz, Q. Ma, P. A. LuValle; Anatomy and Cell Biology, University of Florida, Gainesville, FL

Longitudinal bone growth occurs through endochondral ossification, where cartilage provides the template for ossification into bone. This process is dependent on the proliferation and differentiation of chondrocytes, cartilage cells, to form the ossification template. Activating transcription factor-2 (ATF-2) deficient mice exhibit abnormal growth plates, implicating this protein in modulation of cellular processes necessary for normal bone growth. ATF-2 regulates gene expression by binding the cyclic-AMP response element (CRE) located within promoter regions of target genes. The pocket proteins, retinoblastoma (Rb), p107, and p130, are cell cycle regulators that are potential targets of ATF-2 gene regulation. This study focuses on investigating the expression of the pocket proteins within growth plate chondrocytes and the role of ATF-2 in the regulation of the pocket proteins. Pocket proteins display varying levels of expression within the growth plate as determined by immunohistochenistry. Rb and p130 are expressed at high levels within the wild type mouse growth plate. p107 is expressed at lower levels within the growth plate. Expression of pocket proteins in ATF-2 knockout ($ATF^{m/m}$) mice is altered. Western analysis of protein form wild-type, heterozygous and $ATF^{m/m}$ chondrocytes showed expression of Rb in $ATF^{m/m}$ mice is decreased, while expression of p107 and p130 are increased. Levels of pocket protein mRNAs are also

altered in $ATF^{m/m}$ mice. Transfection of a dominant negative ATF-2 plasmid alters levels of pocket protein mRNAs similar to those observed in $ATF^{m/m}$ mouse chondrocytes. Chromatin immunoprecipitation assays were used to show ATF-2 regulation of pocket protein promoter regions. The loss of ATF-2 leads to abnormal pocket protein expression which may, in turn, lead to cell cycle deregulation, more rapid cell cycle progression and, ultimately abnormal growth plate development.

2034

**Design and Creation of a Floxed Mouse Flightless Gene Targeting Vector**

R. K. Judge,[1] K. Matthei,[2] A. J. Cowin,[3] H. D. Campbell[1]; [1]RSBS, Australian National University, Canberra, Australia, [2]JCSMR, Australian National University, Canberra, Australia, [3]Child Health Research Institute, Adelaide, Australia

The flightless protein is an important member of the gelsolin family of proteins involved in actin remodeling, a process that is central to changes in cell adhesion and motility as well as wound repair processes. Recent studies have demonstrated the primary expression of flightless in proliferative basal and suprabasal keratinocytes. These cell types are responsible for the repair of the epidermis in response to injury. Thus, the objective of this study was to determine the effects of altering the quantity of the flightless protein on wound repair in a gene knockout model through the creation and design of a targeting vector using the Red/ET recombination system. A region of the flightless gene encompassing exons 4 and 5 was flanked by *loxP* sites cloned into adjacent introns. A *Neo* gene cassette for positive selection, flanked by *FRT* sites, was introduced adjacent to the *loxP* site upstream of exon 5, whilst a thymidine kinase cassette for negative selection was introduced downstream of exon 2. Induction of the *Cre* recombinase resulted in the deletion of exons 4 and 5 and the subsequent cryptic splicing of exons 1, 2 and 3 to the downstream introns to produce a null allele. These results demonstrate the successful generation of the flightless null allele. The production of this allele is also the first step towards the construction of a flightless conditional knockout targeted to the epidermis.

2035

**Smooth Muscle Cells (SMC) and Activated Myofibroblasts Employ Distinct but also Common Transcription Control of SM α-Actin Expression**

Q. Gan, T. Yoshida, G. K. Owens; University of Virginia, Charlottesville, VA

SMC and activated myofibroblasts express several common cell selective marker genes such as smooth muscle α-actin (SMα). Indeed, based on these observations, there has been considerable controversy regarding the lineage relationship of these two cell types. Interestingly, results of previous cell culture studies in our lab provided evidence for distinct transcriptional regulatory mechanisms in these cells in that we found mutation of two MCAT elements located at -184 bp and -320 bp in the SMα promoter increased transcriptional activity in cultured rat aortic SMC but had the complete opposite effect of decreasing activity in myofibroblasts. The aim of present study was to determine the role of MCAT elements in control of the SMα transcription in vivo in various cell types. Comparison of transgenic mice containing double-MCAT-element mutant SMα promoter-enhancer LacZ reporter gene (dMCAT-SMα-LacZ mice) versus the wild type SMα promoter-enhancer LacZ (SMα-LacZ) showed no differences of transgene expression in SMC tissues in adult mice. However, of major interest, SMα-LacZ but not dMCAT-SMα-LacZ mice showed induction of transgene within activated myofibroblasts after skin wound injury. In addition, the transgene expression in dMCAT-SMα-LacZ embryos showed a remarkably distinct staining pattern as compared to SMα-LacZ embryos, in that induction of this mutant transgene was developmentally delayed in SMC and completely abolished in cardiac, and skeletal myoblasts, which transiently express SMα during embryonic development. Taken together, these results provide clear evidence that although multiple cell types other than SMC express the SMα gene, they employ distinct transcriptional regulatory controls as compared to mature SMC within adult animals. However, of interest, initial induction of SMα within SMC during early embryonic development appears to require common regulatory controls as in non-SMC including complete dependence on MCAT elements.

2036

**Pitx2, Which Implicated in the Formation of Brachial Arch Arteries, Potently Activates Transcription of Smooth Muscle Marker Genes**

Y. Shang, T. Yoshida, G. K. Owens; Physiology, University of Virginia, Charlottesville, VA

The mechanisms that control early stages of smooth muscle cell (SMC) differentiation are relatively poorly understood. We previously developed an inducible SMC lineage system, an A404 SMC progenitor cell, which activated all of the SMC marker genes after the treatment with retinoic acid (RA). The goal of the present study was to identify novel transcription factors that control early stages of SMC differentiation based on subtraction hybridization screening of RA-treated A404 cells versus untreated A404 cells. Of major interest, we identified over 40 genes that were induced within 48 hours of treatment of RA in differentiated A404 cells. One of these, Pitx2, a homeodomain-containing transcription factor previously shown to play a role in the formation of brachial arch arteries, was transiently induced by 6-fold following RA treatment of A404 cells. Expression of Pitx2 was also induced by 8-fold in an embryonic stem cell-derived embryoid body model of SMC differentiation. Moreover, over-expression of Pitx2 activated transcription of SMC marker genes including smooth muscle (SM) α-actin by 10-fold. In contrast, short interfering RNA-induced suppression of Pitx2 in differentiated A404 SMC cells decreased SM α-actin transcription. Site-directed mutagenesis analysis revealed that the effect of Pitx2 on SM α-actin transcription was mediated by a TAATCC cis-element located at -145 bp and CArG B element at -112 bp of the SM α-actin promoter. Electrophoresis mobility shift assays showed binding of Pitx2 to a TAATCC element. In addition, the interaction between Pitx2 and serum response factor (SRF) was seen by co-immunoprecipitation assays, and co-expression of Pitx2 and SRF synergistically induced SMC marker gene transcription. Taken together, these results suggest that Pitx2 is a novel factor involved in SMC marker gene expression in the early stages of SMC differentiation.

2037

**The Ternary Complex Factor, Elk-1, Plays a Key Role in Modulating Transcriptional Activation of Single and Multiple CArG-Containing Smooth Muscle Cell Marker Genes by Myocardin and MKL Factors**

T. Yoshida, Q. Gan, G. Owens; Physiology, University of Virginia, Charlottesville, VA

Myocardin and its related factors, MKL1 and MKL2, are potent co-activators of serum response factor (SRF). The aims of the present studies were to determine: 1) the role of MKL factors in expression of single CArG-containing, multiple CArG-containing, and CArG-independent smooth

muscle cell (SMC) marker genes; and 2) the mechanisms whereby myocardin and MKL factors selectively induces expression of single CArG-containing SMC marker genes, but not a single CArG-containing c-fos gene. Results showed that expression of MKL1 and MKL2 was unaltered during the induction of SMC differentiation in a novel embryonic stem cell-derived embryoid body model and A404 SMC progenitor cells. Results from gain- and loss-of-function experiments showed that MKL1 and MKL2 regulated the expression of single CArG-containing (e.g. telokin) and multiple CArG-containing (e.g. smooth muscle (SM) α-actin, SM-myosin heavy chain, and SM22α), but not CArG-independent (e.g. aortic carboxypeptidase-like protein and focal adhesion kinase-related nonkinase) SMC marker genes. Mutational analyses of single CArG-containing genes showed that the presence of ternary complex factor (TCF)-binding site adjacent to the CArG element attenuated the responsiveness to myocardin and MKL factors. Chromatin immunoprecipitation assays revealed that one of the TCFs, Elk-1, constitutively bound to the CArG-containing region in the c-fos gene, but not to the CArG-containing region in the telokin gene, and that myocardin selectively enhanced SRF binding to telokin CArG element as compared to c-fos CArG element in SMCs. Results provide novel evidence that MKL factors regulate single and multiple CArG-containing SMC marker genes and that Elk-1 plays a key role in modulating the response of single CArG-containing genes to myocardin and MKL factors. (Supported by grants NIH R01 HL38854, R37 HL57353, and P01 HL19242.)

2038

**Changes in the Muscle Fiber Characteristics of the Anterior Belly of the Digastric Muscle Before and After Weaning in Mice**

K. Sakiyama, M. Yoshii, N. Yanagisawa, S. Abe, Y. Ide; Anatomy, Tokyo Dental College, Chiba, Japan

During the process of growth and development, the digastric muscle is subjected to marked functional changes, including the change from suckling to mastication. In particular, because the anterior belly of the digastric muscle, which is one of suprahyoid muscles, plays an important role in mastication. Therefore, thie muscle seems to be a marked functional change before and after weaning. However, the details remain unknown. Here, in order to clarify the changes in the muscle fiber characteristics of the anterior belly of the digastric muscle before and after weaning, we examined myosin heavy chain (MHC) isoforms at the protein (immunohistochemistry) and mRNA (transcription) levels. As a control, the changes in the muscle fiber characteristics of the sternohyoid muscle, which is anatomically aligned in the same direction as the anterior belly of the digastric muscle, were analyzed. The results showed that, in the anterior belly of the digastric muscle that is involved in mandibular movements in mice, the ratio of a fast-contraction isoform with strong contractile force increased after weaning. We believe that this occurred in response to a functional change from suckling to mastication. On the other hand, there was little change in the composition of sternohyoid muscle.

2039

**Barx2 Regulates Muscle Cell Plasticity**

R. Meech,[1] K. Gonzalez,[1] H. Makarenkova[2]; [1]The Scripps Research Institute, San Diego, CA, [2]The Neurosciences Institute, San Diego, CA

Differentiation and dedifferentiation are fundamental cellular processes mediating muscle development and repair. Recent work has shown that the homeodomain protein Barx2 plays important roles in both of these processes. Overexpression of Barx2 in myoblasts increases expression of some muscle specific genes and accelerates myotube formation, while overexpression in myotubes downregulates muscle genes and promotes dedifferentiation. One potential explanation for this dichotomy is suggested by the domain structure of Barx2: the protein contains both repressor and activator domains that may recruit either co-activator or co-repressor complexes to muscle specific genes in different contexts. Preliminary data suggest that Barx2 helps coordinate cell cycle regulation, cytoskeletal remodeling and muscle specific gene expression during differentiation and dedifferentiation. Thus Barx2 may play an important role in the cellular mechanisms of muscle repair after injury or degenerative disease.

2040

**Properties of Myosin Heavy Chain in Tongue Muscle of Microphthalmic (*mi/mi*) Mice Before and After Weaning**

N. Yanagisawa, K. Sakiyama, S. Abe, Y. Ide; Anatomy, Tokyo Dental College, Chiba City, Japan

To elucidate the effects of teeth on muscle fibers in tongue during the development process, we examined the expression of muscle contractile proteins and the genes for those proteins in normal mice and microphthalmic (*mi/mi*) mice with impaired tooth eruption. The mice were observed during the growth period, including weaning, which is when feeding movements undergo major changes. Expression of the myosin heavy chain (MyHC)-2a protein, whose contraction speed is relatively slow, disappeared after weaning in normal mice, while it remained in high concentrations even after weaning in *mi/mi* mice. The presence of MyHC-2a after weaning in mice with no tooth eruption was attributed to a compensation for lack of proper masticatory function and sucking-like movements, as MyHC-2a is necessary for these movements.

2041

**Role of Laminin-10 in Hair Development**

J. Gao,[1] C. Chen,[1] N. Nguyen,[1] J. Miner,[2] A. E. Oro,[1] M. P. Marinkovich[1]; [1]Epithelial Biology, Stanford University, Stanford, CA, [2]Medicine, Washington University, St. Louis, MO

In the current study we sought to determine the role that laminin-10 plays in hair follicle formation. Lama5 -/- transgenic mice, which lack laminin-10, do not survive beyond E16.5, however development of lama5 -/- skin can be studied after grafting E16.5 day skin to nude mice. While lama5 -/- skin showed a complete lack of hair development after grafting, incubation of lama5 -/- skin in a solution of wild type laminin-10 or recombinant laminin-10 lacking alpha 5 G45 domain prior to grafting restored the development of fully formed hair. However recombinant laminin-10 lacking G3-5 domain showed no ability to support hair follicle formation. These results suggest that the heparin binding domain on the G45 region is dispensable but the integrin binding domain on the G3 region is essential for hair follicle development. E16.5 lama5 -/- skin showed deficient noggin, Msx1 and Lef-1 expression, however Wnts, BMPs, and Msx2 were normal by rt-PCR. By IDIF E16.5 lama5 -/- skin completely lacked noggin expression and failed to show decreased e-cadherin or p-SMAD expression, in marked contrast to wild type skin. Lama5 -/- skin treated with SHH or noggin prior to grafting formed hair follicles, however Wnt3a failed to have any effects. These findings suggest that laminin-10 plays a role in promoting the expression of noggin upon which subsequent steps of phosphoSMAD downregulation, ecadherin downregulation and SHH upregulation occur in the hair morphogenic signaling cascade. Finally, we also found that lama5 -/- epithelial cells failed to support hair development, when mixed with wild type purified dermal papillae cells in hair reconstitution assays. These results implicate laminin-10 as an epithelial derived signal which is required for the hair inducing effects of the dermal papillae.

2042

**A New Chemical Inhibitor of Steroid Biosynthesis**

J. M. Gendron,[1] A. Haque,[2] T. Chang,[1] Z. Wang[2]; [1]Biological Sciences, Stanford/Carnegie Intstitution, Stanford, CA, [2]Plant Biology, Carnegie Intstitution, Stanford, CA

Brassinosteroids (BRs) are ubiquitous plant hormones that control many aspects of plant growth and development. Molecular and genetic tools have been used to delineate the BR biosynthesis and signaling pathways that transduce the BR signal. One important tool that has been used widely in BR research is the small molecule brassinazole (BRZ), which is a triazole-based compound that is thought to inhibit the action of at least one cytochrome p450 enzyme involved in BR biosynthesis. Plants grown on BRZ display typical BR-deficient dwarf phenotypes and can be rescued by exogenous application of BRs, suggesting the compound decreases BR biosynthesis. To identify new inhibitors of BR biosynthesis or signaling, a chemical genetics screen was done using a library of 10,000 small molecules. Dark grown hypocotyl length and expression of a BR signaling marker were assayed to find molecules specifically regulating BR processes. 90 compounds were identified that cause typical BR-deficient phenotypes and affect expression of the BR marker. One of the compounds identified (17) causes phenotypes that can be rescued by application of BR. Compound 17 contains a cyclopropyl side group that is known to react with cytochrome p450s and inhibit their function. Removal of the cyclopropyl group results in loss of activity of the compound, suggesting that the cyclopropyl is necessary for proper function of compound 17. This is the first non-triazole based small molecule identified that inhibits BR biosynthesis. This compound will be a valuable tool to investigate BR signaling.

2043

**Studies on the Expression of SIRT1 (the Human Homologue of SIR2) in HaCaT Cells After Exposure to Caloric Restriction and UV Stress**

C. Dal Farra, E. Roux, E. Bauza, J. Botto, N. Domloge; Vincience, Sophia antipolis, France

Recent studies have established that SIRT1, the human homologue of Sir2, plays an important role in cellular response to stress and aging. It has also been shown that SIRT1 deacetylates p53, preventing cellular senescence and apoptosis induced by stress. Hence, we were interested in studying SIRT1 expression in HaCaT cells, as these cells are commonly used in skin research. SIRT1 and p53 expression in HaCaT cells was evaluated after cells were exposed to caloric restriction or UVB stress. Evaluations were performed through immunostaining, immunoblotting, and mRNA studies. In HaCaT cells irradiated with different doses of UVB, immunoblotting studies revealed an increase in SIRT1 in a dose-dependent manner, up to 40mJ/cm2. Maximum expression of SIRT1 was seen at 30mJ/cm2. Interestingly, p53 expression did not vary significantly, which is consistent with the immortal nature of the cell line. Immunostaining studies revealed the nuclear expression of SIRT1 and confirmed the above results. Furthermore, an increase in SIRT1 mRNA was observed after low UVB doses in mRNA cell studies, and this effect lasted up to 48h after irradiation. Caloric restriction studies showed an increase in SIRT1 expression in a dose-dependent manner with glucose deprivation, and this effect was seen after 48h of deprivation. Interestingly, a significant variation in p53 expression was not observed. These studies demonstrate the expression of SIRT1 in HaCaT cells and reveal the correlation between this expression and UVB and caloric stress. Consequently, these findings strongly suggest that SIRT1 plays an important role in defense against stress and aging in HaCaT cells.

2044

**OsDREB1-A Transcription Factor Gene from Rice, Enhances Cold Tolerance in Tobacco**

G. Zhang, P. Li, F. Chen, C. Quan; Department of Biological Sciences and Biotechnology,Tsinghua University, Beijing, China

The OsDREB1 gene from rice encodes a transcription factor belonging to the DERBP transcription factor subfamily. Many DREBP transcription factors regulate gene expression in response to drought, high-salt, and cold stresses by binding specifically to the dehydration-responsive element (DRE). DRE-binding proteins such as CBF1, DREB1A, and DREB2A have been cloned from Arabidopsis thaliana and have proved to play an important role in stress response of Arabidopsis and several other plants. In this study the OsDREB1 gene was transferred to tobacco plants by the Agrobacterium-mediated transfer method, and 16 transgenic plants were identified, PCR analysis demonstrated that the foreign genes had been integrated into the tobacco genome. Results of freezing stress experiments indicated that the transgenic plants had enhanced cold tolerance.

2045

**Detailed Understanding of Thioredoxin-Linked Redox Regulation by Disulfide Proteome**

H. Yano, S. Kuroda; National Institute of Crop Science, Tsukuba, Japan

Evidences are accumulating to suggest that redox regulations play critical roles in a broad spectrum of biology. Thioredoxin, a ubiquitous 12kDa protein with a catalytically active disulfide bond, plays a central role in controlling the redox status of disulfide bonds in proteins that regulate a range of physiological processes. Recently, Yano *et al.* (Yano, H., Kuroda, S. and Buchanan, B. B., *Proteomics* 2002, *2*, 1090-1096) developed a disulfide proteome technique that visualizes redox change in proteins comprehensively. In this presentation, using disulfide proteome, we examined rice bran and identified embryo-specific protein and dienelactone hydrolase (DLH) as putative targets of thioredoxin. Monitoring both endogenous and recombinant thioredoxin's effects on rice bran proteins and supporting *in vivo* observations propose a mechanism of redox regulation in seed germination, in which thioredoxin activates cysteine protease with a concurrent unfolding of its substrate, the embryo-specific protein, to facilitate degradation. Our findings suggest that thioredoxin controls the lifetime of specific proteins effectively by regulating the redox reactions coordinately. These findings, with the previous observations, suggest the presence of thioredoxin-linked exquisite mobilization mechanisms, in which specific seed proteins are degraded in the respective stages of germination and early seedling development. Also, the regulatory mechanism of rice DLH was found to be different from that of bacterial DLH. The model study demonstrates that the disulfide proteome technique is useful not only to identify targets of thioredoxin, but also to clarify the detailed mechanism of redox regulation. Proteome analysis of regulatory mechanisms, such as those based on phosphorylation and thiol redox status, will add a new dimension to biology.

2046

**Expression and Auxin Effects on the RACK 1 Homolog from Common Bean (*Phaseolus vulgaris*)**

M. A. Villanueva,[1] T. T. Islas-Flores,[1] I. R. Islas-Flores,[2] E. L. Bearer[3]; [1]Plant Molecular Biology, Institute of Biotechnology-UNAM, Cuernavaca, Morelos, Mexico, [2]Plant Biochemistry, Centro de Investigación Científica de Yucatán, A.C., Mérida, Yucatán, Mexico, [3]Biology and Medicine,

Brown University, Providence, RI
Germination and growth in plants is a natural event that involves multiple molecular reactions within complex and highly regulated biochemical cascades which will eventually develop into a photosynthetic, autotrophic plant. The cytoskeleton and signal and adaptor proteins play key roles during these early stages and most of the plant life cycle. One of these proteins of the adaptor type is the receptor for activated protein kinase C (RACK1). The plant homolog was partially sequenced by RT-PCR using mRNA from common bean embryo axes, and an antibody against an internal peptide was raised in rabbits. Western blot analyses indicated that the protein was present in detectable amounts in the embryo axes, and that the protein was present in both microsomal and soluble fractions from this tissue. Expression analysis during germination revealed that the protein was present at relatively constant levels from 0 to 96 h. However, the mRNA showed a peak of maximal expression at 36h. The presence of auxin during germination only delayed the maximal peak of expression of the mRNA to 48 h but had no effect on the protein level. An auxin blocker, naphtylphtalamic acid had no effect on either mRNA or protein expression levels. Southern blot analysis showed the presence of a small gene family with only one or two members. These data indicate that the RACK1 homolog from bean is present in soluble and insoluble forms in the bean embryo axis, and that its mRNA has an important peak of expression at times when germination stops and growth and elongation start in the seedling, but external auxins or auxin blockers have a minimal effect on this phenomenon.

## Growth Factors in Development (2047-2057)

2047

### Epidermal Growth Factor (EGF) Regulates Differentiation of Small Gaba-ergic Cortical Neurons

E. C. Walcott,[1] K. Crossin,[1,2] H. Makarenkova[1]; [1]The Neurosciences Institute, San Diego, CA, [2]The Scripps Research Institute, San Diego, CA
The mechanisms underlying the development of neuronal diversity are largely unknown. In the mammalian cerebral cortex Gamma-aminobutyric acid (GABA) neurons represent 10-25% of all neurons. Neuronal cultures prepared from rat embryonic cerebral cortex at E 16-18 form two different populations of GABA-ergic neurons. A distinct population of large GABA-ergic neurons was found in the cultures just after the plating. This population differentiates into large neurons with extensively developed and branched dendritic trees and is responsible for generation of synchronous oscillatory network activity. The ability of these neurons to differentiate even in the presence of mitogen inhibitor ARAC suggests that most of these neurons are post mitotic. Neurons in the second population of GABAergic cells are much smaller, predominantly bipolar in appearance and they form clusters between large neurons. Small GABA-ergic neurons are generated in culture during the first week and their function and mechanisms underlying their differentiation are unknown. A further characterization of small GABA-ergic neurons shows that they extensively express epidermal growth factor receptor (EGFR), while the expression of fibroblast growth factor receptors FGFRs is low. In correlation with this finding the application of FGFR inhibitor SU5402 to the cortical cultures just after plating had little effect on differentiation of small GABA-ergic neurons while treatment with EGFR inhibitors PD153035 and ZD 1839 almost completely eliminated population of small GABA-ergic neurons. These treatments did not affect the differentiation of large GABA-ergic neurons however application of FGFR inhibitor disturbed synchronous oscillatory network activity of these cells. These findings suggest that EGF may be involved in differentiation of cortical neuronal subpopulations later in development.

2048

### Brain-Derived Neurotrophic Factor Potentiates EphrinA2-Induced Neuronal Growth Cone Collapse

C. Mays, D. S. Ice, E. V. Wong; Biology, University of Louisville, Louisville, KY
Ephrin ligands and their cognate Eph receptor tyrosine kinases play an important role in many developmental processes including angiogenesis, early tissue organization, and axon guidance. Within one of the classic models for the study of axon guidance, the developing chick retinotectal projection, ephrinA2 and ephrinA5 have well-described expression patterns and roles in the optic tectum. EphrinA2, expressed in a smooth anterior-posterior gradient across the tectum, in combination with graded nasotemporal expression of its receptor in the retina, provides positional cues to aid in precise retinotopic mapping of retiinotectal synapses. EphrinA5, expressed in a sharp gradient in just the posterior third of the tectum may also provide some positional information, but likely acts primarily to prevent overshoot of axons. As the retinal axons approach their targets, they begin to receive trophic support via brain-derived neurotrophic factor (BDNF). It was therefore hypothesized that BDNF may modulate ephrin sensitivity to help direct synaptogenesis at the correct site. By testing chick retinal ganglion cell axons and growth cones in vitro, it was found that 18-hr pretreatment with BDNF potentiates growth cone collapse induced by ephrinA2, but not ephrinA5. Upregulation of EphA receptors for ephrinA2 is one possible mechanism for the potentiation by BDNF. Immunohistochemical investigation of this possibility has revealed a significant increase in EphA3 receptor expression in retinal neurons treated with BDNF compared to those that have grown in a control nutrient medium, suggesting that this is part of a mechanism to regulate ephrin- mediated axon guidance by neurotrophic factors.

2049

### VEGF Acts as a Guidance Signal on Cerebellar Granule Cell

N. G. Thomasset,[1] P. Salin,[2] N. Chounlamountri,[1] M. Belin,[1] C. Ruiz de Almodovar,[3] L. Moons,[3] P. Carmeliet,[3] C. Meissirel[1]; [1]Faculty of Medicine, INSERM 433, Lyon, France, [2]Faculty of Medicine, UMR5167 CNRS, Lyon, France, [3]University of Leuven, Flanders Interuniversitary Institute for Biotechnology, Leuven, Belgium
Migration of undifferentiated neural progenitors is critical for the development and repair of the nervous system. However, mechanisms and factors that regulate migration of these progenitors are not well understood. Our objective is to investigate the role of Vascular Endothelial Growth factor (VEGF-A), a major angiogenic factor, during postnatal cerebellar development when proliferation or migration/axogenesis of granular precursor is extensive. Our results showed that VEGF is strongly expressed in developing Purkinje neurons with VEGFR2 receptor expression restricted to granular cell precursors in the external granular layer. Using purified cultured cells obtained from P5 cerebellar cortices, we studied VEGF effect on cerebellar granule cell proliferation, migration and axonal outgrowth in presence or not of a specific VEGFR2 inhibitor, SU1498. Quantification of proliferating granule cell precursors labelled with Bromodeoxyuridine showed a significant dose effect of VEGF up to a 25% increase in proliferation. We investigated VEGF effect on directed cell migration using an in vitro chemotaxis migration assay and co-cultures. Cell counts and axonal area measurements indicate that axons and cell bodies were chemoattracted to short- and long-range VEGF signals, through stimulation of

VEGFR2. Such directed processes could involve local guidance effects at the growth cone level. Interestingly, we demonstrated using a standard chemotropic assay, that axonal growth cones of isolated granule cells turned towards a VEGF gradient. Furthermore, disruption of local VEGF gradient in the cerebellum of VEGF?/?knockin mice impaired inward migration of granule cells. In conclusion, our results demonstrate that VEGF acts as a regulatory signal to control cell proliferation and directed migration as well as axogenesis during postnatal cerebellar development, emphasizing that common mechanisms underlie vessels and nerves guidance

2050

**Hb-egf Promotes Epithelial Cell Migration In Eyelid Development**

N. Mine, R. Iwamoto, E. Mekada; Cell biology, Microbial desease Osaka University, Suita, Japan

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family of growth factors that binds to and activates the EGF receptor (EGFR) and ErbB4. Here, we show that HB-EGF-EGFR signaling is involved in eyelid development. HB-EGF expression is restricted to the tip of the leading edge of the migrating epithelium during eyelid closure in late gestation mouse embryos. Both HB-EGF null (HB del/del) and secretion-deficient (HB uc/uc) mutant embryos exhibited delayed eyelid closure due to slower leading edge extension and reduced actin bundle formation in migrating epithelial cells. No changes in cell proliferation were observed in these embryos. In addition, activation of EGFR and ERK was decreased in HB del/del eyelids. Crosses between HB del/del mice and waved-2 mice, a hypomorphic EGFR mutant strain, indicate that HB-EGF and EGFR interact genetically in eyelid closure. Together with our data showing that embryos treated with an EGFR-specific kinase inhibitor phenocopy HB del/del embryos, these data indicate that EGFR mediates HB-EGF-dependent eyelid closure. Finally, analysis of eyelid closure in TGF- a null mice and in HB-EGF and TGF-a double null mice revealed that HB-EGF and TGF-a contribute equally to and function synergistically in this process. These results indicate that soluble HB-EGF secreted from the tip of the leading edge activates the EGFR and ERK pathway, and that synergy with TGF-a is required for leading edge extension in epithelial sheet migration during eyelid closure.

2051

**A Novel G Protein-Coupled Receptor Controls the Cortical Assembly in Early Xenopus Embryos Through Small RhoGTPases**

Q. Tao, C. Wylie; Developmental Biology, Cincinati Childrens Hospital, Cincinnati, OH

The actin skeleton plays critical roles in all multicellular organisms. However, the mechanism that controls the assembly of cortical actin, and its remodeling during embryogenesis, are poorly understood. We have been using early Xenopus embryos to study these processes. We have previously shown that the Armadillo-repeat protein, plakoglobin, is both necessary and sufficient for cortical actin assembly in early Xenopus embryos, and is down stream of a small Rho GTPase, cdc42. In order to find more components of this pathway, we carried out an expression screen, using an egg cDNA library. This screen identified a novel G-protein-coupled receptor (GPCR) related to the GPR4 subfamily. We have named this receptpr Xflop. Both gain- and loss-of-function assays showed that Xflop-mediated signaling is both necessary and sufficient for assembly of the actin skeleton in the early Xenopus embryo. In particular, Xflop is required for regulating the overall amount of cortical actin. Epistasis showed that RhoA, cdc42, and Rac1 are all required for the full function of Xflop in regulating cortical actin assembly in early Xenopus embryos. Our work highlights the importance of intercellular signaling in controlling the overall amount and density of the cortical actin network in early embryos.

2052

**Expression Pattern of Serotonin Signaling Components during Chick Gastrulation**

T. Fukumoto, T. Mikawa; Cell & Developmental Biology, Cornell University Medical College, New York, NY

We have previously shown that both agonists and antagonists of serotoninergic signaling can randomize the expression of laterality genes during early embryogenesis. While the data suggested a novel role of serotoninergic signaling in left-right axis formation, it is unclearwhen and how the lateral patterning is influenced by this signaling. In the present study, we examined expression profiles of enzymes that regulate the level of serotonin (5-HT). These included tryptophan hydroxylases (TPH1 and TPH2), which synthesize 5-HT from tryptophan, and monoamine oxidase (MAO), which degrades 5-HT. Our RT-PCR analysis detected these three enzymes in both pre-gastrula and gastrula stage embryos. Our in situ hybridization analysis detected th preferential expression of these thee enzymes at the posterior region of the embryonic disc, including Koller's sickle, at stage XII/XIII. After H.H.stage 2, their expression was detected in the primitive streak. Consistent with these molecular data, our immunohistochemical analysis detected 5-HT in a posterior midline region of the embryonic disc at stage XIV and in the primitive streak at H.H.stage 3. These results are consistent with a model where serotoninergic signaling plays a regulatory role in laterality patterning. The data also suggest that left-right axis formation begins perhaps at much earlier developmental stage than previously thought. (Supported in part by the NIH)

2053

**Quantitative Assessment of Tubulogenesis in vitro Effects of Growth Factors**

P. Nelson, N. Ratkevicius, S. Sepulveda, C. Araya, J. Garrido; Cell & Molecular Biology, Catholic University of Chile, Santiago, Chile

A rat gastric epithelial cell line (ERG-2) established in our laboratory grows as stable monolayers in high-serum (10% FBS) media. In low-serum (2.5% FBS) media these cells undergo tubular morphogenesis to produce branched structures overlying the monolayer. While characterizing this process we observed that cell subpopulations seen as distinct foci undergo morphological changes that precede tubulogenesis. We have shown such changes accompany epithelial-mesenchymal transition on localized groups of cells that divide more actively than the polygonal cells in the underlying monolayer, and that such increased proliferation is independent of MEK1-2 activity. To understand the possible roles of growth factors in the regulation of this process we developed a method that allowed us to assess tubulogenesis quantitatively. We have employed this method to evaluate the effects of several growth factors and inhibitors of intracellular signaling cascades. Our results show that both EGF and HGF are powerful inductors of tubulogenesis, while incubation with TGFbeta inhibits both spontaneous tubulogenesis and the stimulatory effect of EGF and HGF on this process. On the other hand, inhibition of MEK1-2 results in increased tubulogenesis, while inhibition of PKA, PI3K and ROCK abrogate the stimulatory effects of EGF and HGF. These results indicate that multiple, diverse signaling pathways are involved in the regulation of this process.

2054

**Effect of Thrombin Peptide TP508 on Chondrocyte Differentiation in Mouse Embryonic Limb Bud Cells and Ex Vivo Cultures of Mouse embryo Metatarsals**

S. S. Dong,[1] R. Serra,[1] G. M. Fuller,[1] D. H. Carney[2]; [1]Dept of Cell Biology, Univ. Alabama, Birmingham, AL, [2]Dept Human Biological Chem and Genetic, Univ. Texas Medical Branch, Galveston, TX

The thrombin peptide, TP508 (Chrysalin®), is a synthetic peptide representing the domain of thrombin required for high-affinity binding to a specific class of cell surface thrombin receptors. This peptide has been shown to promote or accelerate repair and regeneration of soft and hard tissues including cartilage. TP508 is now in human clinical trials to evaluate its potential use in fracture repair. Little is known, however, about the mechanisms by which TP508 stimulates repair and regeneration of a specific tissue such as cartilage. We utilized embryonic limb cell micro-mass and metatarsal organ culture models to evaluate the ability of TP508 to stimulate specific phases of osteochondral differentiation. Addition of TP508 to micro-mass cultures of limb cells isolated from 11.5 day embryos resulted in a dose dependent increase in Alcian blue proteoglycan staining after 3 days in culture. An increase in the total number of chondrogenic nodules was not observed suggesting TP508 does not recruit cells to the chondrogenic lineage but directly regulates proteoglycan synthesis. TP508 also resulted in an increase in cell proliferation after 6 days in culture. A scrambled TP508 peptide had no effect on these cells. Metatarsal bones from 15.5 day embryos were then cultured in the presence of 100µg/ml of TP508 or Scrambled TP508. Cultures treated with TP508 demonstrated an expansion of the hypertrophic zone relative to cultures treated with scrambled peptide. Alterations in the total length of the bone were not detected. This observation suggests that TP508 may activate a set of signals that leads to hypertrophic differentiation in chondrocytes. These studies support the potential use of TP508 for applications involving both bone and cartilage repair. (Supported by NIH AR46982, AR45605, and OrthoLogic Corp).

2055

**Wnt5a Exhibits a Growth Inhibitory Effect on the Development of the Mammary Gland**

K. Roarty, R. Serra; Cell Biology, University of Alabama at Birmingham, Birmingham, AL

Postnatal mammary gland development involves a complex interplay between epithelial and stromal components to regulate development and function of the gland. Wnt proteins represent a family of signaling molecules that participate in diverse developmental processes in embryonic and adult tissues. Wnt5a is expressed during multiple phases of mammary gland development; however, its role remains to be elucidated. In vitro studies have suggested Wnt5a mediates adhesion and migration of mammary epithelial cells. To investigate the role of Wnt5a in the development of the mouse mammary gland in vivo, Wnt5a slow-release plastic pellets containing 170ng Wnt5a were implanted into the glands of 3-5 week old virgin Balb/C mice. Control pellets containing BSA were implanted into the contralateral glands. Ten days after implantation mice were injected with 60µg/g body weight BrdU 2 hours before sacrifice, at which point glands were collected for wholemount and histological analysis. Evaluation of Wnt5a-implanted wholemounts revealed an inhibition of the ductal tree through the fat pad vs. those glands receiving BSA implants. In addition, the terminal end buds of the glands receiving the Wnt5a implants were noticeably smaller in size compared to control glands. BrdU analysis revealed that this inhibition and size difference was due to a decrease in proliferation in the terminal end buds in those glands receiving the Wnt5a implants. TUNEL analysis showed no difference in the number of apoptotic cells between Wnt5a and control glands. These results demonstrate that Wnt5a inhibits the progression of the terminal end buds through the fat pad during puberty accompanied by a decrease in size of the end bud, and that this effect is due to a decrease in proliferation and not an increase in apoptosis.

2056

**Lipopolysaccharide Inhibits FGF-10 Expression in Fetal Mouse Lung Mesenchyme**

J. T. Benjamin, R. Smith, N. Davis, L. S. Prince; Pediatrics, University of Alabama at Birmingham, Birmingham, AL

Prenatal exposure to inflammation increases the risk of bronchopulmonary dysplasia in preterm infants. The lungs of infants with bronchopulmonary dysplasia have fewer, more simplified alveoli, possibly due to defective distal airway branching. We have previously shown that *E. coli* lipopolysaccharide (LPS) decreases distal airway branching in fetal mouse lungs. FGF-10 is a master regulator of branching morphogenesis and is expressed in the distal lung mesenchyme. We hypothesized that LPS decreases FGF10 expression leading to altered distal airway branching in the fetal mouse lung. We first demonstrated that FGF-10 could regulate distal airway branching. In our fetal mouse lung explant model, recombinant FGF-10 increased the number of distal airway branches from 40.2 branches/mm$^2$ to 49.6 branches/mm$^2$ ($p<0.05$). Addition of a polyclonal anti-FGF10 antibody inhibited distal airway branching (30.2 branches/mm$^2$, $p<0.01$); anti-FGF-10-treated explants resembled those exposed to LPS. Using real-time PCR, we measured decreased FGF-10 gene expression in LPS-treated fetal lung explants (55 % of control, $p<0.05$). LPS also decreased the expression of the FGF-10-induced genes BMP4, BMPR1a, and PERP. LPS did not alter FGF-10 expression in explants from TLR4 mutant mice. These data suggested that LPS altered gene expression along the FGF-10 signaling pathway. To further test our hypothesis, we restored distal airway branching in LPS-treated explants by adding exogenous FGF-10. TGFβ1 is a known suppressor of FGF-10 expression. However, LPS did not increase TGFβ1 gene expression or increase the amount of soluble TGFβ1 released from cultured fetal lung explants. Therefore, LPS appeared to decrease FGF-10 expression independent of TGFβ1. The decrease in FGF-10 expression resulted in defective distal airway branching and suggests a mechanism by which innate immune signaling can alter lung development.

2057

**Roles of Notch Signaling in Chondrogenesis of Mouse Mesencephalic Neural Crest Cells**

M. Ota, K. Nakanishi, Y. Chan, K. Ito; Department of Biology, Osaka University, Toyonaka, Japan

Neural crest cells respond to various signaling molecules and differentiate into a wide variety of cell types, including melanocytes, peripheral neurons and their glial cells, and smooth muscle cells. In addition, cranial neural crest cells give rise to chondrocytes, osteobalsts, and components of the craniofacial skeleton. It has been shown that FGF2 promotes chondrogenesis of cranial neural crest cells. Furthermore, this signaling molecule activates Notch signaling in development of the nervous system. In the present study, therefore, we have examined the roles of Notch signaling in chondrogenesis of mouse mesencephalic neural crest cells. The activation of Notch signaling by the addition of a Notch ligand, Delta, promoted chondrogenesis in the neural crest cell cultures. Notch activation or FGF2 exposure during the first 24 hours in culture was indispensable for an increase of the number of cells expressing type 2 collagen that is a specific marker of chondrocytes. The expression of SOX9, which is a

member of SRY-like HMG box-containing gene family and a transcription activator of type 2 collagen, was upregulated by FGF2 treatment, while BMP4 had no effects on the expression of type 2 collagen or SOX9. Furthermore, the treatment with Delta induced the expression of Sox9. The present data show that chondrogenesis of the neural crest cells is promoted by the activation of Notch signaling as well as FGF2 treatment. Thus, Notch signaling may play important roles in differentiation of mouse mesencephalic neural crest cells into chondrocytes.

## Stem Cells I (2058-2077A)

2058

### Nonhuman Primate (NHP) Embryonic Stem Cell Lineage Contributions in Mouse:NHP Interspecific Chimeric Blastocysts

C. Simerly, J. Park, E. Park, D. McFarland, H. Qidwai, E. Jacoby, C. Navara, G. Schatten; Pittsburgh Development Center, Pittsburgh, PA

Embryonic stem (ES) cell pluripotency, convincingly demonstrated in reaggregated embryos in which the resultant offspring have ES cell contributions to all germ layers and tissues, including the germ line, has only been achieved using mouse embryonic stem (mES) cells. Overwhelming ethical concerns preclude attempts with human embryonic stem (hES) cells. Projects utilizing NHP's might address crucial questions surrounding the clinical utility of hES cells. Here, we investigate both intra-specific (NHP-ESC: NHP embryo) and inter-specific (NHP-ESC: Mouse embryo) reaggregated embryos to explore nhpES cell lineage contributions to the resultant chimeric blastocysts. For intra-specific chimera, NHP-ES cells, aggregated either with zona-free, CellTracker™ Orange-labeled fertilized rhesus embryos or injected (<10 ES cells) into the perivitelline space of 6-8 cell stage embryos, were cultured to the blastocyst stage or transferred to timed recipients for offspring production. For inter-specific chimera, NHP-ES cells were aggregated with zona-free fertilized (2N) or tetraploid (4N) mouse 2-cell stage embryos and grown to the blastocyst stage. Mouse embryo +YFP-labeled mouse ESC chimera served as controls. All blastocysts were analyzed dynamically and after fixation using pluripotency markers. Preliminary results show NHP-ESCs integrate into NHP embryos to form chimeric morulae, although a pregnancy has yet to be established. Conversely, NHP-ESCs aggregated with mouse 2N or 4N embryos do not integrate into the inner cell mass (ICM) in resultant blastocysts, suggesting inter-specific primate-rodent aggregations do not form chimeric embryos. We conclude that intra-specific, but not inter-specific, chimera aggregations will be useful for ethically addressing questions of primate chimeric pluripotency.

2059

### Differentiation-Related Changes in Mitochondrial Properties Might be an Indicator of Stem Cell Competence

T. A. Lonergan, C. A. Brenner, S. Rajasekaran, B. D. Bavister; Biological Sciences, University of New Orleans, New Orleans, LA

Several methods can be used to assess stem cell competence, including the expression of cell surface markers and telomerase activity. We hypothesize that mitochondrial characteristics might be a faster way to verify stem cell competence. Several properties of a multipotent monkey mesenchymal cell line, which has previously been shown to differentiate into adipocytes, chondrocytes and osteocytes, change with increasing passage number. Cells from the earliest passage (P11) vs. those from later passages (15 - 17) are characterized by : a) a much higher percentage of cells (85% vs. 18%) with a perinuclear arrangement of mitochondria when labeled with Mitotracker; b) a much lower percentage of cells (1% vs. 57%) with a fused mitochondrial arrangement, in which mitochondria appear to coalesce into larger aggregates; c) a much lower percentage of cells with lipid droplets (1% vs. 36%) indicating differentiation into adipocytes; and d) an eleven-fold increase in ATP content per cell (0.45 vs. 4.49 pmoles ATP/cell, P=0.012). The percentage of cells with lipid droplets correlates positively to the percentage of cells with a fused mitochondrial arrangement ($r^2$ = 0.999, P = 0.019), suggesting that this mitochondrial distribution may be a marker for differentiation into adipocytes. Collectively, these data suggest that the presence of a perinuclear arrangement of mitochondria, accompanied by a low ATP/cell content might be a valid indicator of stem cell competence, while departure from this profile indicates cells are differentiating. Similar mitochondrial properties will be examined in the future using other primate adult and embryonic cell lines.

2060

### Human Embryonic Stem Cells Show Highest BrdU Uptake and Apoptosis on Low Density Feeder Cells in Culture

J. A. Ozolek,[1] E. Jane,[2] P. J. Sammak,[3] G. P. Schatten[3]; [1]Pathology, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Neurosurgery, Children's Hospital of Pittsburgh, Pittsburgh, PA, [3]Pittsburgh Development Center, Pittsburgh, PA

During human development and particularly the developing nervous system, cell proliferation and programmed cell death occur in concert as tissue and organogenesis proceed. The objective was to determine cell culture conditions that promote or discourage proliferation and apoptosis of human embryonic stem cells (hESCs). HSF-6 hESCs were grown in Knockout DMEM on CF-1 mouse feeders or U87 glioblastoma cells at densities of 50,000 (low), 100,000 (intermediate), and 150,000 (high) cells per well for 7, 12, and 19 days. Cells were incubated with BrdU one hour prior to fixation. BrdU and TUNEL staining was performed according to manufacturer protocol (Roche Diagnostics Incorporation, Indianapolis, IN) and examined by confocal microscopy. Positive BrdU and TUNEL cells were found primarily at edges and between established colonies. Many positive BrdU cells demonstrated bright intensity signal at edges of nuclei. At day 7, both feeders produced high numbers of BrdU positive and TUNEL positive cells compared to day 12 and day 19. Numerous BrdU positive cells were seen on low density U87 feeder conditions at days 7, 12, and 19 compared to intermediate or high density. Day 7 U87 feeder conditions produced higher numbers of BrdU positive cells at all densities compared to either day 12 or 19. We conclude hESCs grown on U87 feeder conditions demonstrate greater numbers of apoptotic cells than cells grown on CF-1 feeders. HESCs on U87 feeders show greater numbers of BrdU positive cells consistently over time. Independent of the feeder system, proliferation and apoptosis may be positively correlated. We speculate that the lower density feeder systems may induce cell cycle and programmed death sequences due to low amounts of specific factor (s). U87 cells may supply additional factors that promote cell turnover.

2061

### Focused Laser Beams as Versatile Tools for Stem Cell Purification and Clonal Expansion

A. Buchstaller, A. Buchstaller, S. Soria-Lopez, S. Sagebiel, R. Huss; Pathology, University of Munich, Munich, Germany

Stem cells have the potential to differentiate into specialized cells from various lineages. Differentiation is often associated with changes in morphology and cell surface marker expression. To understand the intrinsic molecular events associated with differentiation, it is important to gain pure populations of cells and to analyze their behavior and characteristics. The selection and isolation of cells with specific features also stand at

the center of stem cell therapy and tissue engineering. Heterogeneity of cell preparations poses a serious problem to the molecular analysis and therapeutic use of stem cells. We used "Laser Microdissection and Pressure Catapulting" (LMPC, P.A.L.M. Microlaser Technologies) for the isolation of life stem cells according to cell morphology and marker expression. We first isolated single cells from 1) murine embryonic carcinoma cell lines, 2) murine adult stem cell lines and 3) preparations of human bone marrow mononuclear cells. The recultivated clonal cells expand rapidly, express the same stem cell-specific markers as the originating cells and maintain the same morphology. We then proceeded to sort out and clonally expand single adherent fibroblast-like CD29+/CD44+ mesenchymal stem cells from a complex mixture of bone marrow-derived cells. In a different set of experiments we separated undifferentiated embryonic carcinoma cells from their differentiated progeny after treatment with all-trans retinoic acid. Cells exhibiting a neuronal morphology including neurites and growth cones were isolated with LMPC, recultivated, and analyzed for the expression of the growth-associated molecule GAP-43 and activity of L-type calcium channels. Applied in a high throughput fashion, coupled to single cell manipulation and high content single cell analysis, LMPC will prove itself as an important tool in basic research, drug development, and regenerative medicine.

2062

**Chromatin-related Proteins in Pluripotent Mouse Embryonic Stem Cells Are Downregulated after Removal of Leukemia Inhibitory Factor**

A. Kurisaki,[1] T. S. Hamazaki,[2] M. Asashima,[3] H. Sugino[1]; [1]Division of Molecular Cytology, The Institute for Enzyme Research, The University of Tokushima, Tokushima, Japan, [2]Department of Tissue Regeneration, Research Institute International Medical Center of Japan, Tokyo, Japan, [3]Department of Life Sciences, The University of Tokyo, Tokyo, Japan

Embryonic stem (ES) cells have generated enormous interest due to their capacity to self-renew and the potential for growing many different cell types in vitro. Leukemia inhibitory factor (LIF), bone morphogenetic proteins, octamer binding protein 3 or 4, and nanog are important factors in the maintenance of pluripotency in mouse ES cells. However, the mechanisms by which these factors regulate the pluripotency remain poorly understood. To identify other proteins involved in this process, we did a proteomic analysis of mouse ES cells that were cultured in the presence or absence of LIF. More than 100 proteins were found to be involved specifically in either the differentiation process or the maintenance of undifferentiated state. Among these, chromatin-related proteins were identified as the major proteins in nuclear extracts of undifferentiated cells. In contrast, endothelial and mesenchymal markers, as well as some actin-related proteins were induced during the differentiation following LIF withdrawal. These results suggest that specific chromatin-related proteins may be involved in maintaining the unique properties of pluripotent ES cells. And moreover, we could demonstrate that the proteomic approach is a powerful method to elucidate the mechanism of pluripotency and differentiation process in the stem cell.

2063

**Response of Human Embryonic Stem Cells [hESCs] to Ionizing Radiation-induced DNA Damage Includes Rapid Cell Death or Immediate Differentiation**

O. Momcilovic,[1,2] J. Mich-Basso,[3,2] C. J. Redinger,[3,2] A. Ben-Yehudah,[3,2] G. Schatten,[3,2] C. S. Navara[3,2]; [1]Human Genetics, University of Pittsburgh, Pittsburgh, PA, [2]Pittsburgh Development Center, Pittsburgh, PA, [3]Ob/Gyn and Reproductive Sciences, University of Pittsburgh, Pittsburgh, PA

Human embryonic stem (hES) cells exhibit heterogeneous and exaggerated responses to ionizing radiation induced DNA damage. In our study we employed gamma irradiation of hES cells and somatic cells, followed by live cell imaging, time course recoveries and immunocytochemistry by confocal microscopy. After gamma irradiation WI-38 human embryonic lung fibroblast cells halted progression through the cell cycle and continued with normal mitotic divisions one-day post-irradiation. Conversely, pluripotent hES cells responseded in a varied fashion with cells in the center of large colonies dying beginning within 2 h, so that the center of colonies appeared hollow by 6 h after irradiation. Cells on the periphery of the colony did not undergo cell death but adopted a differentiated phenotype. In contrast, small colonies retained their integrity at all times and seemed only to differentiate. Human embryonic cells returning to mitosis after one day displayed mostly abnormal mitotic spindle morphologies. Expression of the pluripotency marker Oct-4 began disappearing almost immediately after irradiation and did not return until about 1 week later as detected by confocal microscopy. At approximately the same time normal colonies reappear, suggesting that some cells escape either cell death or differentiation fates and can return to an undifferentiated phenotype. Nuclear localization of phopho-p53 increased dramatically after irradiation. This effect was much more pronounced than in WI-38 cells and contrasts reports of poor nuclear localization of p53 in mouse embryonic stem cells. These results suggest that even colonies expressing uniform markers of pluripotency are heterogeneous and that hESCs human embryonic stem cells have alternative responses to DNA damage as compared to than do somatic cells.

2064

**Isolation and Characterization of STAT3 Target Genes in Mouse Embryonic Stem (ES) Cells**

Y. Na,[1] Y. Song,[2] S. Hong[1,2]; [1]Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Republic of Korea, [2]School of Biological Sciences, Seoul National University, Seoul, Republic of Korea

Mouse ES cells can be maintained as a pluripotent, self-renewing population in the presence of leukemia inhibitory factor (LIF). It has been reported that STAT3 activation is sufficient to maintain an undifferentiated state of mouse ES cells. However, the downstream effectors of LIF/STAT3 pathway are poorly understood. To isolate and characterize the genes that are regulated by STAT3 in undifferentiated ES cells, we performed chromatin immunoprecipitation (X-ChIP). We were able to isolate several candidates whose expressions are regulated by STAT3. The expression of FoxJ1, a member of forkhead box family of transcription factor, was increased after the removal of LIF or the treatment of Jak2 inhibitor, AG490. Using EMSA analysis, it was found that STAT3 could bind to the promoter region of FoxJ1. These suggest that FoxJ1 is regulated by LIF/STAT3 signaling pathway. Another candidate gene, ZFP-57, a putative zinc finger protein, was decreased after the induction of differentiation. Using EMSA analysis, we found that STAT3 could bind to the promoter region of ZFP-57 and regulate directly the expression of ZFP-57. Mouse ES cells transfected with ZFP-57 could maintain an undifferentiated state a little bit longer than the wild type counterpart in the absence of LIF, which suggests that ZFP-57 might be the key regulator of ES cell self-renewal.

2065

**STAT3 Regulates the Differentiation of Mouse ES Cells into Neural Precursor Cells**

K. M. Foshay, G. Gallicano; Cell Biology, Georgetown University, Washington, DC

While the JAK/STAT signaling cascade plays a major role in the onset of gliogenesis, its functions during early neuronal development and differentiation of neural precursor cells are unclear. STAT3 is both present and active as early as E10.5 in the developing mouse CNS. Because gliogenesis does not begin until several days later, E14, the presence of STAT3 at E10.5 suggests that JAK/STAT signaling is functioning earlier in neural development. However, data produced by a neural specific STAT3 knockout mouse contradict the idea that STAT3 functions early in neural development, as these mice do not exhibit any overt neural phenotypes. We suggest a possible solution to the ambiguity of STAT3 function in neural development by demonstrating that the true function of STAT3 lies in regulating the differentiation of embryonic stem cells into neural precursor cells. This fate choice occurs prior to E10.5 and prior to the deletion of STAT3 in the neural specific knockout model. To test our hypothesis, we generated STAT3 dominant negative stem cells and subjected them to neural differentiation induced by retinoic acid. When compared to controls, STAT3 dominant negative ES cells differentiated into significantly fewer neural precursor cells, as determined by expression of nestin. Due to the decrease in neural precursor cells, STAT3 dominant negative cultures also expressed drastically lower levels of neural specific markers, such as Emx2, Synaptophysin, and the astrocyte marker, s100b.

2066

**Feasibility of RNAi Mediated Inhibition of BMP Signaling During Maintenance and Neural Differentiation of Mouse Embryonic Stem Cells**

L. Bjorklund, T. Andersson; Karolinska Institute, Center for Genomics and Bioinformatics, Stockholm, Sweden

BMP signaling has been shown to regulate stem cell maintenance and proliferation as well as differentiation of mouse embryonic stem cells (mESC). Furthermore, BMP-antagonism is essential for neuronal differentiation within the adult neural stem cell niche. However, the ESC response to BMPs may vary at different time-points during differentiation. Thus, it is of importance to be able to study the effects of BMP inhibition at various stages of neural differentiation from mESCs. Using lipid based transfection of undifferentiated ES cells, we were able to achieve over 90% decrease in the expression level of the Bmp4 gene at 24 hrs post-transfection. To achieve transfection of adherent cells undergoing neural differentiation as monolayers, we used a capillary based electroporation system to deliver the siRNA molecules. Preliminary results suggest that it is possible to deliver siRNA also to adherent mESCs undergoing neural differentiation as monolayers. We conclude that it is possible to introduce siRNA against molecules such as BMPs into mESCs both as undifferentiated cells undergoing passage as well as during differentiation into neurons.

2067

**Wnt Drives In Vitro Development of Dorsal Mesoderm from Embryonic Stem Cells in the Absence of BMP**

M. Tanaka,[1] Y. Wang,[1] C. Wood,[1] N. Brouard,[1] D. Haylock,[2] P. Simmons,[1] N. Nakayama[2]; [1]Peter MacCallum Cancer Institute, Melbourne, Australia, [2]Australian Stem Cell Centre, Peter MacCallum Cancer Institute, Melbourne, Australia

Various mesodermal cell-types, defined by the expression of VEGFR2 (Flk-1), PDGFRα and/or N-cadherin (Ncad), are induced in vitro from embryonic stem (ES) cells in a serum-free medium with or without BMP4. BMP4 affects regional specificity during early embryogenesis. Therefore, we investigated the effect of BMP4 on region-specific mesoderm formation during ES cell differentiation. $Ncad^+Flk\text{-}1^-PDGFR\alpha^+$ cells, phenotypically identical to those previously isolated from BMP4-containing culture and determined to be osteochondrogenic and non-hemogenic, were produced without BMP4 or even in the presence of BMP-inhibitor, noggin. They were also osteochondrogenic and non-hemogenic. T and Tbx6 genes were transcribed in both $Ncad^+Flk\text{-}1^-PDGFR\alpha^+$ cells, suggesting BMP4-independent mesoderm formation. On the contrary, BMP4 was essential for the hemogenic $Ncad^+Flk\text{-}1^+PDGFR\alpha^-$ cell-genesis. Regardless of the exogenous BMP4, production of $Flk\text{-}1^+$ and $PDGFR\alpha^+$ mesodermal cells was completely abolished by Wnt-inhibitor, Dkk1 or Fzd8CRD-Fc, and was upregulated by purified Wnt3a protein. Global gene-expression profiling suggested that the $Ncad^+Flk\text{-}1^-PDGFR\alpha^+$ cells developed without BMP4 contained somitic mesodermal cells (expressing Meox1/2, Pax1/3, Praxis), but those generated with BMP4 did not. Transcripts of marker genes for the anterior-distal region of the embryo appeared during the first 5 days of differentiation without BMP4, while many of them did not with BMP4. In fact, without BMP4, neural tube marker genes (Pax6, Sox1), and anterior visceral endoderm marker genes (Cer1, sFRP1) were detected. However, posterio-proximal site marker genes (Wnt3, T, MesP1) were expressed preferentially in the presence of BMP4. Thus, 1) Wnt signaling appears to be essential for $Flk\text{-}1^+$ and $PDGFR\alpha^+$ mesoderm formation from ES cells, 2) exogenous BMP4 seems to impose proximal region-specific characteristics on the developed progenitor cells, and suppresses anterior-distal characteristics, and 3) thus, paraxial (dorsal) mesoderm-like cells are selectively generated in the absence of BMP4.

2068

**Molecular Commitment to Osteogenic Lineage of Human Umbilical Cord Blood Derived Mesenchymal Stem Cells**

P. A. Jaramillo,[1] J. P. Morales,[1] J. J. Minguell,[2] A. A. Erices[1,2]; [1]Departamento de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile, [2]Clínica Las Condes, Santiago, Chile

Mesenchymal stem cells (MSC) represent a subpopulation of cells present in the bone marrow stroma (BM-MSC) and umbilical cord blood (UCB-MSC). They are characterized for their capacity to differentiate along the osteogenic, adipogenic, chondrogenic and myogenic differentiation pathways, among others. The aim of this work was to characterize the expression of osteogenic markers in MSC obtained from BM and UCB, and to evaluate comparatively their ability to respond to an osteogenic differentiation stimuli. It was found that cultures of both cellular populations express early differentiation markers towards the osteogenic (Runx-2/Cbfa-1, Msx-2), adipogenic (PPAR-γ), condrogenic (Sox-9), miogenic (MyoD, Myf-5), pancreatic (Pdx-1, Ngn-3, Isl-1) and neural (NeuroD, Nestin) differentiation pathways. When osteogenic differentiation was induced, both cell types did not show significant variations in the expression levels of Runx-2/Cbfa-1 and Msx-2. However, after this induction, Msx-2 expression showed a translocation to cytoplasmatic domains, which was increased with the progression along the osteogenic differentiation. This pattern was more accentuated in UCB-MSC than in BM-MSC. When analyzing the expression and/or activation of intermediate/late osteogenic markers, it was found that UCB-MSC showed a smaller proportion of alkaline phosphatase positive cells and absence of expression of

osteopontin in comparison to BM-MSC. In response to the osteogenic stimuli, UCB-MSC increased alkaline phosphatase activity to a greater degree in comparison to BM-MSC. On the other hand, both cellular populations express osteopontin in response to the osteogenic differentiation stimuli. These results suggest that both UCB-MSC and BM-MSC represent multipotential cellular populations and that UCB-MSC have a greater proportion of immature osteoprogrenitor cells, with a different potential to the osteogenic differentiation pathway.

2069

**Isolation, Growth and Differentiation Characteristics of a Novel Human Mesenchymal Stem Cell Source from Umblical Cord Stroma**

S. Karahuseyinoglu,[1] O. Cinar,[1] F. Kara,[2] A. Can[1]; [1]Histology-embryology, Ankara University School of Medicine, Ankara, Turkey, [2]Zubeyde Hanim Maternity Hospital, Ankara, Turkey

Mesenchymal stem cells are of great interest in the past decades although they are far from being well-characterized cells unlike the hematopoietic ones. Few studies revealed a potential source of mesenchymal stem cells in the connective tissue stroma of the umbilical cord (UC). The purpose of this study is to isolate stem cells from human UC stroma and to assess the cellular characterization of these cells throughout their differentiation into different lineages. UCs were obtained from Cesarian sections (n=6) performed following uncomplicated, term pregnancies. Stromal tissues excluding blood vessels was digested and then resuspended pellet was plated into culture dishes as 20 cells/cm$^2$. Upon reaching to 80-90 % confluency (4000 cells/cm$^2$), cells were subcultured and used for cell dynamics (viability, doubling time, mitotic index), differentiation experiments and microscopic observations at certain time points. Synchronously, cells in culture were used for certain protein quantification. Morphological appearance of cells differed in concordance with the number of subcultures. While during the first 10-20 division cycles cells were strongly positive for vimentin (marker for mesodermal origin) and pancytokeratin (marker for epidermal origin), pancytokeratin positivity gradually declined and totally diminished when cells began to form multicellular bodies usually after 60-70 division cycles. Contact inhibition, anchorage dependency and arrest in mitosis after serum deprivation strongly ruled out the possibility of a tumorigenic phenotype. Cells at early division cycles subjected to neuronal differentiation gained typical neuronal appearance. Additionally, they successfully differentiated into mesenchymal lineages such as osteogenic, chondrogenic and adipogenic cells in conditioned cultures. Taken together, overall data led us to suggest that human UC stromal cells bear multi-germ layer characteristics, therefore have a great range of differentiation capacity and may be considered as a new source for stem cells.

2070

**Neuroendocrine Potential of Human Umbilical Cord Blood Derived Mesenchymal Stem Cells**

J. P. Morales,[1] N. McConnell,[1] P. Jaramillo,[1] A. Fierro,[2] J. J. Minguell,[2] A. A. Erices[1,2]; [1]Departamento de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile, [2]Clínica Las Condes, Santiago, Chile

Adult pancreas neogenesis and embryonic stem cell differentiation to insulin producing cells (IPC) require cells expressing neuroendocrine genes like *nestin*, *neuroD*, and *neurogenin-3*. Both postnatal bone marrow and umbilical cord blood contain mesenchymal stem cells (MSC) expressing nestin, suggesting a neuroendocrine potential, but this capacity is unknown. With the purpose of generating new cellular therapies for diabetes, it has been our principal interest to study the differentiation potential of mesenchymal stem cells (MSC) derived from human umbilical cord blood or bone marrow as possible progenitors of pancreatic endocrine tissues or IPC. We focused on characterizing the presence of molecular markers of endocrine pancreas lineage in MSC such as Nestin, Pdx-1, Neurogenin-3, Islet-1, Pax-4, and Insulin that suggests these cells as good candidates to generate IPC. In addition, we study the characterization of cells of pancreatic acinar tissue since it has been documented that cells derived from this fraction could also participate in the generation of IPC. MSC were characterized on expression of mesenchymal markers as SH2, SH3, SH4, and smooth muscle actin-α and their capacity to differentiate to osteogenic, chondrogenic and adipogenic lineages. Through analysis by RT PCR, immunoflorescence, and western blot, the results of this first stage are promising and showed that both MSC and acinar cells express pancreatic markers with a slight variation in the expression levels. Coculture of MSC on acinar cells increased expression level of markers as Pdx-1 in MSC, a essential gene for pancreas development and regeneration. Based on this, we propose that under the appropriate stimuli our MSC could differentiate in cells that generate insulin. We can conclude that MSC unbilical cord blood and bone marrow represent, at a molecular level, a potential precursor of IPCs under the correct stimuli.

2071

**Inhibition of Osteogenic Differentiation in Human Mesenchymal Stem Cells by Exogenous c-myc-tagged Focal Adhesion Kinase**

W. A. Williams, N. E. Schapiro, S. R. Christy, R. F. Klees, G. L. Weber, G. E. Plopper; Biology, Rensselaer Polytechnic Institute, Troy, NY

Osteogenic differentiation of human mesenchymal stem cells (hMSC) occurs in vitro upon binding to collagen I in the absence of soluble osteogenic stimuli. We hypothesize that activation of integrin-associated protein focal adhesion kinase (FAK) via phosphorylation of its tyrosine-397 is one of the primary intracellular signals that leads to osteogenic differentiation of hMSC. To test our hypothesis, we infected hMSC with a retrovirus containing a c-myc epitope-tagged FAK wild type gene (c-myc-FAK) or a c-myc epitope-tagged FAK Y397F mutant gene (c-myc-FAK F397), and identified and isolated infected hMSC by their resistance to the antibiotic G418. A statistically significant increase in the phosphate mineral-to-matrix ratio was observed in hMSC infected with a virus containing the gene coding for c-myc-FAK when plating on collagen I as compared to cells infected with a virus containing the gene coding for c-myc-FAK F397 or a virus containing no c-myc-FAK gene. Variable results were obtained when immunoblotting for the c-myc epitope tag. However, expression of c-myc-FAK and c-myc-FAK F397 in infected hMSC was investigated using immunofluorescence and RT-PCR. Moreover, expression of phenotypic markers of osteogenesis in these cells was investigated using immunoblotting, immunofluorescence, and RT-PCR. These data could demonstrate a key role for ECM-induced FAK signaling in osteogenic differentiation of hMSC.

2072

**Differentiation of Human Embryonic Stem Cells into Neuroblasts Expressing the Migration Marker, Doublecortin**

J. A. Ozolek,[1] E. Jane,[2] P. J. Sammak,[3] G. P. Schatten[3]; [1]Pathology, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Neurosurgery, Children's Hospital of Pittsburgh, Pittsburgh, PA, [3]Pittsburgh Development Center, Pittsburgh, PA

In the context of brain regeneration and repair, in particular damage to the fetal brain or premature infant brain, it will be useful to know how

culture conditions can produce specific phenotypes of cells. In the developing nervous system, the expression of doublecortin (DCX) signifies the presence of migrating neurons. Doublecortin is a microtubule binding protein required for normal neural migration and cortical layering during development. The objective of this study was to determine if differentiation into neuronal and glial phenotypes is promoted by 1) varying feeder density, 2) using different feeder layers and 3) to determine phenotypic changes over time in culture. HSF-6 hESCs were grown in cell culture on CF-1 mouse feeder layers or U87 glioblastoma cells at densities of 50,000, 100,000, and 150,000 cells per well. Cells were grown in Knockout DMEM media, fixed and double-labeled with antibodies to nestin (intermediate filament expressed in neuroepithelium) and doublecortin and examined by confocal microscopy. Comparison of DCX expression was done between HSF-6 on CF-1 and U87 at day 19 only. At day 19, hESCs grown on CF-1 feeders at 100,000 cells/well demonstrated numerous cells primarily at the edge of colonies with strong DCX and nestin coexpression compared to U87 at 100,000 cells/well that showed weaker expression, fewer colonies, and some loss of nestin coexpression. Comparing expression of hESCs grown on U87 only, cells at 100,000 cells/well and fixed on day 12 demonstrated the most cells at the edge of colonies expressing both nestin and DCX compared to day 7 or 19. We conclude that intermediate density feeder cell conditions promote the differentiation of hESCs in culture into cells with neuroblast/neuroepithelial qualities. CF-1 feeders may promote this differentiation more efficiently than U87 glioblastoma cells.

2073

**Differentiation of Human Adult-Derived Stem Cells into Neuronal Cells**

D. Coffey, J. M. Thompson; Biology, California State University, San Bernardino, CA

Stem Cells are characterized by their ability to reproduce themselves for extended periods, and by their unique ability to give rise to cells of distinct and varying lineages. This ability to transform into disparate cells and subsequent tissue holds great promise for clinical application of stem cells in both replacement and restorative therapies. In 1998, Human Embryonic Stem Cells (ESC) were isolated and shown to be pluripotent, as they were capable of differentiation into all tissues types. More recently, stem cells have been isolated from adult tissues. Adipose, blood, muscle and bone tissue have all been found to contain a subset of undifferentiated cells which can be directed by endogenous growth factors to restore function or regenerate tissue. In our study, we have employed adult-derived stem cells obtained from Human bone marrow stromal cells. These Mesenchymal Stem Cells (MSC) have been maintained in vitro as undifferentiated cells for multiple passages, demonstrating the proliferative abilities ascribed to stem cells. Further, we have used specific induction protocols to produce differentiation, or possibly an initial de-differentiation into a precursor cell and subsequent differentiation, of these MSCs into cells displaying a distinctly neuronal phenotype. These cells exhibit morphologies that are, at once, drastically different from their stromal cells progenitors and strikingly reminiscent of neuronal phenotypes complete with long neuritic processes. Immunocytochemical analysis revealed expression of the neural stem cell marker Nestin and the presence microtubule associated protein (MAP) in the post-induction population and a lack of these results in the pre-induction population. These results indicate that there exists an endogenous population of adult stem cells in many different mesenchymal tissue types that can be induced to differentiate into cells similar to those tissues or into cells of endodermal or even ectodermal origin.

2074

**Electron Microscopic Observation of Rat Perirenal Adipose Tissue and Adipogenesis in Vitro**

K. Han,[1,2,3] S. Kim,[1,2,3] C. Park,[4] C. Uhm[1,2,3]; [1]Department of Anatomy, Korea University College of Medicine, Seoul, Republic of Korea, [2]Brain Korea 21 Biomedical Sciences, Seoul, Republic of Korea, [3]Korea Lung Tissue Bank, Seoul, Republic of Korea, [4]Electron Microscope Facility, Korea University College of Medicine, Seoul, Republic of Korea

Adipose tissue contains many stem cells of mesenchymal origin that are able to self-renew and capable of differentiation along multiple lineages. To obtain the basic structural information of the adipose tissue and adipogenic stem cells, we observed perirenal adipose tissue and cultured adipocytes of rats by electron microscopy. After anesthetization of male Sprague-Dawley rats, perirenal adipose tissues were dissected out, and stem cells were isolated and cultured with DMEM with 10% FBS and antibiotics at 37°C in 5% $CO_2$ incubator. Formation of fat droplets was observed after Oil-Red-O staining. For electron microscopy, cultures and adipose tissues were fixed in 2.5% glutaraldehyde buffered with 0.1M phosphate buffer (pH 7.4), postfixed with 1% osmium tetroxide and processed for scanning and transmission electron microscopy according to the routine protocols. Platinum-coated samples were observed with a FE-SEM (Hitachi S-4700), double-stained with uranyl acetate and lead citrate sections were observed with a TEM (Hitachi H-7500). In adipose tissue, adipocytes formed a group separated by intervening connective tissue septa. Each adipocyte was surrounded by a meshwork of fine reticular fibers, and adjacent cells were connected by a dense network of fibers. Capillaries were present in the connective septa. Measurements of diameter revealed two populations of adipocytes; small of 18.4 um in diameter and large of 73.8 um in diameter. The nature of this subpopulation is not known. Preadipocytes were mainly spindle-shaped at first and became round as small lipid droplets were accumulated. The small fat droplets gradually fused together to form one large fat droplet filling up to 80% of the cytosol in the mature adipocyte. Our results confirmed the general architecture of the adipose tissue and the differentiation of preadipocytes into adipocytes. Further study in the obesity and metabolic diseases is needed.

2075

**Adult Stem Cells from Adipose Induce Neural Differentiation of Human Embryonic Stem Cells**

L. E. Kokai,[1] K. G. Marra,[1] J. P. Rubin,[2] E. Jane,[3] J. A. Thaller,[3] G. P. Schatten,[3] P. J. Sammak[3]; [1]Bioengineering, University of Pittsburgh, Pittsburgh, PA, [2]Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, [3]Ob/Gyn/Reproductive Sciences, Pittsburgh Development Center of Magee-Women's Research Institute, Pittsburgh, PA

A current barrier for human embryonic stem cells (hESCs) in clinical use is the requirement of animal products for differentiation to defined fates with exposure to nonhuman pathogens and immunogens. This study used human adipose stem cells (hASCs) as feeder cells in lieu of animal cells, thus providing a xeno-free co-culture system. hASCs are adult meshenchymal stem cells, are easily obtained, have a known immunosuppressive ability and are similar to currently employed mouse preadipocytic stromal cells used for neuronal differentiation. hASCs were isolated from adipose tissue collected from patients undergoing elective surgical procedures and characterized by fluorescence activated cell sorting. hESCs, line HSF-6 and H7 were maintained following the provider's recommendations and then co-cultured for 19 days on either mitomycin-C treated hASCs or human fibroblasts. Immunocytochemical analysis showed hESCs cultured with hASCs were Oct4 negative and Pax6, A2B5, GFAP, nestin, and

beta-III tubulin positive in a density dependent manner. Astrocytes (GFAP) were dominant at low hASC density and neurons (nestin, beta-III tubulin) increased at higher hASC density. Results were similar for two hESC cell lines on hASC lines derived from three unique patients. hESCs cultured with fibroblasts as a control sporadically expressed mature neural markers, few astrocyte markers and retained undifferentiated cell populations. At 60 days, complex neural networks were produced on high-density hASCs. hASCs have several advantages: Teratoma forming cells were not detected past 19 days. hASC produced either high yield astrocytes or supported extensive neuronal outgrowth. Patient-specific hASCs transplanted with neural stem cells may reduce graft vs. host rejection, or modify the endogenous neural stem cell niche to promote neurite outgrowth. These findings recommend hASCs as in vitro feeder layers or in vivo niche support cells in the treatment of neurodegenerative diseases by cell therapy.

2076

**Differentiation of Human Adult Adipose-derived Stem Cells into Neurons *in vitro***

V. Nguyen,[1] J. M. Thompson,[1] J. Shores[2]; [1]Biology, California State University, San Bernardino, CA, [2]Plastic Surgery, Loma Linda University Medical Center, Loma Linda, CA

One of the hottest areas in research today is stem cell research. Stem cells are undifferentiated cells that can develop into any type of cell in the body. Researchers believe that stem cells can revolutionize medicine by enabling physicians to repair or replace damaged organs or tissues. Adipose tissue has been shown to contain multipotent stem cells called human adipose-derived stem cells (HuADSC). These cells are capable of differentiating into any of the three germ layers: ectoderm, endoderm and mesoderm. In our research, we isolated the HuADSC's that were obtained from human liposuction debris. HuADSC cultures were established and grown for 4 passages in RPMI medium with 10% fetal calf serum and 1% antibiotic/antimycotic. We treated our cells with 20 ng/ml basic fibroblast growth factor (bFGF), 0.5 µM retinoic acid (RA) or combinations of these in hoping to differentiate them into neurons that can be used as therapeutic treatments for diseases involving the central nervous system (CNS) or the peripheral nervous system (PNS). RA is commonly used in embryonic stem (ES) cell differentiation. ES-cell-derived 3D cell aggregates called embryoid bodies treated with RA have been shown to give rise to excitable and functional neurons. Other studies showed that induction of ES cells with FGF can differentiate into specific neurons such as dopaminergic neurons. Morphological changes were analyzed with a Nikon Eclipse TS100 microscope and captured with NIH Image. Our results show that bFGF is the best inducing factor. Morphological changes including neurite-like branching were observed in our treated cultures. Furthermore, anti-MAP (marker for mature neurons) and anti-Nestin (marker for neural stem cells) antibodies were used to confirm the differentiation of cells.

2077

**Osteogenic Differentiation of Adipose Derived Mesenchymal Stem Cells from Adult Human Lipoplasty**

D. Williams,[1] J. M. Thompson,[1] J. Shores[2]; [1]Biology, California State University, San Bernardino, CA, [2]Plastic Surgery, Loma Linda University Medical Center, Loma Linda, CA

Stem cells are undifferentiated cells that during embryogenesis give rise to the three germ layers ectoderm, mesoderm, and endoderm. The ability to recover and manipulate these cells has become a major focus of research in the scientific community. Recent studies have demonstrated the presence of adult mesenchymal stem cells (MSC) in the supportive matrix of bone marrow and adipose tissues. The ability of adult stem cells to regain their plasticity by differentiating into multiple tissue types including osteogenic, chrondrogenic, myogenic and adipogenic tissue, promises to be powerful force for treatment of disease pathology. The procedures for inducing differentiation have not been fully established, nor have methods of inoculation *in vivo* for the purpose of tissue engineering and vascular regeneration. In this study we propose to establish a successful induction medium for osteogenic differentiation. Adipose derived stem cells (ASC) were isolated from human lipoplasty (liposuction). Lipoaspirate cells were harvested by washing with phosphate buffered saline followed by treatment with collagenase. Cells were then grown to confluence in a control medium of RPMI, 10% fetal calf serum and 1% antibiotic/antimycotic. Induction of cells was initiated by replacing control medium with an osteogenic medium consisting of RPMI, 10% FCS, 1% antibiotic/antimycotic, 0.1 µM dexamethasone, 50 µg/ml ascorbic acid, and 10mM β-glycerophosphate. Following differentiation, cells were analyzed for morphological changes. Images were viewed using bright field and phase contrast microscopy. Alzarin red stain was used confirm the presence of extracellular matrix calcification. Immunochemistry was performed to demonstrate the production of alkaline phosphate. The ultimate goal of this research is to develop not only a standard for the induction medium but also to develop a 3-dimensional scaffold combining multiple tissue types capable of successful inoculation *in vivo* in a clinical setting.

2077A

**Cord Blood Derived Embryonic Stem Cells**

C. P. McGuckin,[1] L. Denner,[2] M. Baradez,[3] S. Navran,[4] J. Zhao,[2] R. Urban,[2] R. Tilton,[2] N. Forraz[1]; [1]Regenerative Medicine, Clinical Laboratory Sciences, University of Newcastle, Newcastle upon Tyne, United Kingdom, [2]Internal Medicine-Endocrinology & Stark Diabetes Center, University of Texas Medical Branch, Galveston, TX, [3]Stem Cell Therapy Programme, Kingston University, Kingston upon Thames, London, United Kingdom, [4]Synthecon Corporation, Houston, TX

**OBJECTIVES**: The lobby for Embryonic Stem Cells (ES) is strong and highly visible. But when will these cells be a routine clinical intervention? ES limitations are clearly cellular immunogenicity and amount of producible homogeneous tissue - and without feeder layers! Adult stem cells (ADS) from Umbilical Cord Blood (UCB) however, propose a huge untapped source with the global yearly birth rate at 100 million, with added advantage of long telomere and ontogenically. We report here reproducible harvest and development of UCB cells with ES cell markers ["Cord Blood derived embyonic-like stem cells" (CBE's)] and developed them for defined Tissue Engineering. **METHODS**: A CBE harvest and expansion: UCB [elective caesarian birth] was separated by sequential immunomagnetic removal [nucleated granulocytes, red cells, haemopoietic myeloid / lymphoid progenitors]. After 7 days high density culture in microflasks, [$10^5$ cells/ml, IMDM, FCS10%, Thrombopoietin 10ng/ml, Flt3-Ligand 50ng/ml,c-kit ligand 20ng/ml], CBE colonies formed adherent to the substrata, maintaining for 6 weeks, subcultured and continued minimum >13 weeks. B CBE Liver Tissue Engineering: CBE's were cultured with hepatic growth medium [IMDM, FCS10%, HGF 20ng/ml, bFGF 10ng/ml, EGF 10ng/ml, c-kit ligand 10ng/ml] in a NASA-derived Synthecon microgravity rotating bioreactor. Rotational and volume considerations produced a low shear stress environment with optimal gas exchange. **RESULTS:** CBE's were positive for TRA-1-60, TRA-1-81, SSEA-4, SSEA-3 and Oct-4, but not SSEA-1, indicating restriction to the human stem cell compartment. Tissue Bioreactor engineered CBE's

developed positivity for hepatic markers (week 4) Cytokeratin-18, Alphafetoprotein, Albumin. Cohesive tissue constructs developed. **CONCLUSION:** CBE's are a viable alternative for stem cell research and defined tissue engineering and appropriate for clinical translation protocols.

## Synapse Formation & Function II (2078-2095)

2078

### Ubiquitin Dependent Proteolysis of Rapsyn and its Physiological Significance

K. Min, S. Nam, J. Lee; Biological Sciences, Seoul National University, Seoul, Republic of Korea

Rapsyn (Receptor-Associated Protein at the SYNapse) plays an important role in acetylcholine receptor (AChR) clustering, which is required for normal neuromuscular transmission. Rapsyn is co-transported, and probably pre-assembled, with the receptors before the functional receptors are transported to the plasma membrane. Both overexpression and knock-down of rapsyn result in the failure of AChR clustering *in vivo*. In a search for the regulatory mechanism of rapsyn abundance in *C. elegans*, we found that RPY-1, the rapsyn homolog, is ubiquitinated and degraded by proteolysis. RNA*i* analyses revealed UBC genes for rapsyn degradation. In addition, genes involved in neddylation were required for rapsyn degradation, suggesting that a cullin-containing E3 ligase complex is involved in this process. We identified the adaptor protein between the cullin protein and its substrate rapsyn. Knockdown of any gene involved in RPY-1 degradation caused higher stability of rapsyn even under the normal condition, suggesting that the cullin complex system functions for quality control of the components in a protein complex in the physiological conditions. We then identified a human homolog of the BTB-containing adaptor for rapsyn. We found that this human homolog interacts with and ubiquitinates rapsyn together with the cullin E3 ligase. We mapped the domains within rapsyn and the BTB-containing protein required for their interaction. Our data raises the possibility that genes involved in rapsyn degradation may be causal genes for the congential myasthenic syndrome in humans.

2079

### Interdomain Interactions in the Tumor Suppressor Discs Large Regulate Binding to the Synaptic Protein GukHolder

Y. Qian, K. E. Prehoda; Department of Chemistry, Institute of Molecular Biology, Eugene, OR

Membrane-associated guanylate kinase (MAGUK) is a large family of scaffolding proteins found in many signal pathways at the cell membrane in multi-molecular organisms. Here we show that binding of the synaptic protein GukHolder (GukH) to Discs Large (Dlg), a member of MAGUK proteins, is actively regulated through complex interdomain interactions. GukH binds to a composite binding site on the SH3 and Guanylate Kinase-like (GK) domains of Dlg, but this binding site is obscured by an intramolecular interaction between the SH3 and GK domains. A protein interaction domain, PDZ, adjacent to the SH3 domain relieves this inhibition. The protein CRIPT, which links Dlg to the microtubule cytoskeleton, binds the PDZ domain and causes a conformational change in the PDZ-SH3-GK cassette that obscures the GukH binding site. The PDZ-SH3-GK module is a common feature of MAGUK scaffolds. Our results indicate that the complex multi-domain architecture of scaffolds can be used to actively regulate the proteins that they associate with.

2080

### CRF and Urocortin Differentially Modulate GluRdelta2 Expression and Distribution in Parallel Fiber-Purkinje Cell Synapses

N. Gounko,[1] V. Rybakin,[2] D. Kalicharan,[1] Z. Siskova,[3] A. Gramsbergen,[4] J. J. L. van der Want[1]; [1]Cell Biology, University of Groningen, Groningen, The Netherlands, [2]Institute of Biochemistry I, University of Cologne, Cologne, Germany, [3]Membrane Cell Biology, University of Groningen, Groningen, The Netherlands, [4]Medical Physiology, University of Groningen, Groningen, The Netherlands

Stabilization and maturation of synapses is crucial for neuronal circuit formation. Early in development, an overabundance of synapses is formed, followed by selective maintenance or elimination of specific synapses and ultimately resulting in the formation of correct and durable connections. The ionotropic glutamate receptor GluRdelta2 is highly expressed in cerebellar Purkinje cells and plays a role in the differential distribution of parallel fiber and climbing fiber synapses on Purkinje cell dendritic spines. However, the mechanisms responsible for the distribution of the GluRdelta2s in the postsynaptic membrane are not well understood. Here, we analyze the effect of corticotropin-releasing factor (CRF) and urocortin (UCN), two closely related peptide regulators, on the GluRdelta2 expression in Purkinje cells and its distribution in Purkinje cell spines' postsynaptic membranes in *in vitro* culture. Using real time PCR, we establish that both CRF and UCN do indeed modulate the expression of GluRdelta2 gene, but only urocortin treatment results in an increased GluRdelta2 protein level, suggesting a posttranscriptional mechanism of its regulation. We further observed different immunogold patterns of GluRdelta2 distribution in the PSD. UCN treatment provides a linear distribution, whereas CRF treatment results in a clustered distribution of GluRdelta2 along the PSD, probably through the regulation of the submembrane cytoskeleton. We propose a model explaining these functional differences based on differential receptor preference and signaling cascade activation by CRF and UCN. We hypothesize that CRF and UCN may induce local dendritic instability, allowing activity-dependent morphological changes in Purkinje dendritic spines resulting in global effects on synaptic transmission and plasticity.

2081

### Control of Neuronal Morphogenesis and the Delta/Notch Signalling Pathway by Shrub, a Coiled-Coil Protein Involved in Multivesicular Body Formation

N. T. Sweeney,[1] J. E. Brenman,[2] Y. N. Jan,[2] F. Gao[3]; [1]Gladstone Institute of Neurological Disease and Neuroscience Graduate Program, UCSF, San Francisco, CA, [2]Department of Physiology and Howard Hughes Medical Institute, UCSF, San Francisco, CA, [3]Gladstone Institute of Neurological Disease, Neuroscience Graduate Program and Dept. of Neurology, UCSF, San Francisco, CA

Dendritic branching is a complex process controlled by many signaling pathways, including Notch. Here we report the identification of mutations in the *shrub* gene that led to increased dendritic branching and enhanced signaling by the Notch pathway. Using multiple dendritic (MD) sensory neurons in *Drosophila* larvae and the MARCM technique, we found that neurons with *shrub* mutations showed ectopic dendritic and axonal branching, indicating a cell-autonomous function in neuronal morphogensis. *Shrub* encodes a novel coiled-coil protein homologous to yeast protein Snf7. Snf7 is involved in the formation of a subset of late endosomal compartments known as the multivesicular bodies. *Shrub* mutations caused

abnormal distribution of late endosomal markers. In addition, the expression levels of Notch intracellular domain (NICD) and Notch ligand Delta, as well as Notch-dependent transcription, were elevated in *shrb* mutant embryos. Genetic studies reveal a novel cell-autonomous function for Delta in promoting dendritic branching. These data support a model in which regulation of dendritic morphogenesis by *shrb* involves endosomal modulation of the Delta/Notch signaling pathway.

2082

**Association of PrPc with Syntaxin 1A and Hsp40**

K. Beck, J. E. A. Braun; Physiology and Biophysics, University of Calgary, Calgary, AB, Canada

Cellular prion proteins ($PrP^c$) are glycoproteins expressed on the cell surface of many cell types, but are predominantly found in the CNS, localized to pre- and post-synaptic membranes. The cellular function of $PrP^c$ at these sites remains largely unresolved. Here we sought to establish whether direct *in-vitro* protein-protein interactions could occur between $PrP^c$ and SNARE proteins (syntaxin 1A, VAMP and SNAP25), which mediate synaptic vesicle fusion at the pre-synaptic plasma membrane, and also the molecular co-chaperones CSP and Hsp40. Our results revealed novel interactions between $PrP^c$ and both syntaxin 1A-GST and Hsp40-GST at 4 and 37 $^oC$. Interactions between $PrP^c$ and Hsp40-GST were specific and concentration-dependent. Conversely, interactions between $PrP^c$ and immobilized syntaxin 1A were biphasic and were blocked in the presence of VAMP and SNAP25. Our findings suggest a role for $PrP^c$ in modulating the synaptic vesicle exocytotic machinery as well as proteins that are crucial for recovery from stress-induced protein damage.

2083

**Long-Lasting Filopodial-like Protein Complexes Induced by Repeated Cocaine Administration in the Rat Nucleus Accumbens**

S. Toda, H. Shen, S. Cagle, P. W. Kalivas; Neurosciences, Medical Univ. South Carolina, Charleston, SC

Addiction to cocaine is associated with persistent changes in synaptic function, especially in medium spiny neurons of nucleus accumbens (NAc). Given that actin-based ultrastructual change of spine underlies cocaine-induced neuronal plasticity, just as the case of long-term potentiation or long-term depression, we investigated the effect of acute or repeated cocaine on actin cycling and actin-regulating proteins in the rat NAc using subcellular fractionation. Acute and chronic cocaine administration produced reversible and enduring, respectively, elevations in F-actin in the NAc. Acute cocaine administation induced F-actin accumulation in association with the increase of p-cofilin. Inhibition of p-cofilin by a cell-permeable TAT-cofilin peptide did not decrease the level of F-actin, however, it promoted the recruitment of ARP3 and PSD-95 in postsynaptic density (PSD), suggesting a transition to a lamellipodia-like structure by inhibiting LIMK1-cofilin cascade. Repeated cocaine administration followed by a 3-weeks withdrawal maintained the upregulated level of F-actin in parallel with continuous increase of Mena, p-VASP and p-cortactin at tyrosine 412, although the level of LIMK1 and p-cofilin was downregulated. Microinjection of latrunculin A in the NAc rapidly and reversibly disrupted F-actin as well as many PSD proteins in the repeated cocaine animals, but not in control animals. Taken together, it is concluded that repeated cocaine administration induces an enduring filopodial spine morphology containing mature PSD protein complex in the NAc and that this actin-based ultrastructure is maintained by enhanced actin turnover.

2084

**Long Term Culture of Adult Rat Spinal Cord Cells in a Novel Serum-Free Medium and on a Synthetic Organosilane Substrate: A Morphological, Immunocytochemical and Electrophysiological Characterization**

M. Das, N. Bhargava, S. Patil, S. Seal, J. J. Hickman; University of Central Florida, Orlando, FL

In this work, we documented by morphological analysis, immunocytochemistry and electrophysiology, the development of a culture system that promotes the growth and long-term survival of dissociated adult rat spinal cord neurons and glial cells. This system is comprised of a patternable, non-biological, cell growth promoting organosilane substrate N-1[3-(trimethoxysilyl)propyl]-diethylenetriamine (DETA), coated on a glass surface and an empirically derived novel serum-free medium, supplemented with specific growth factors[1]. The culture consists of 60% neurons and 40% glia. Neurons were characterized by immunoreactivity for neurofilament150, neuron specific enolase, MAP-2, beta-tubulin, NeuN, ISLET-1, choline acetyltransferase(ChAT), Vesicular Glutamate Transporter 1(VGLUT1), Vesicular GABA Transporter(VGAT) and MO-1 antibodies. The glial cells were characterized by immunoreactivity for GFAP, O1, O4, myelin basic protein and OX-42 antibodies. Electrophysiology experiments indicated that 60% of the cells expressed voltage dependent inward and outward currents similar to the neurons and were able to generate single, double and multiple (repetitive firing) action potentials. 40% of the cells either showed inward, outward or very small inward and huge outward currents and these recordings are similar to the recording documented for the glial cells in the literature. The cultures were maintained for 8-10 weeks. This novel culture system will be a useful tool to study adult mammalian spinal cord cell patterning, electrophysiology, repair, myelination, degeneration and to screen different putative drug candidates for spinal cord repair and degenerative diseases of the spinal cord such as Multiple Sclerosis and Amyotrophic Lateral Sclerosis. Reference: 1. Adult rat spinal cord culture on an organosilane surface in a novel serum-free medium. In Vitro Cell Dev Biol Anim (In press) 2005 Das M, Bhargava N, Gregory C, Riedel L, Molnar P, Hickman JJ.

2085

**A Role for the C elegans L1CAM Homologue, LAD-1/SAX-7, in Tissue Attachment and Potential Synapse Function**

X. Wang, L. Chen; GCD, University of Minnesota, Minneapolis, MN

Cell adhesion molecules belonging to the L1 family (L1CAMs) are highly conserved proteins implicated in vertebrate and invertebrate nervous system development. We are carrying out a genetic analysis of the *C. elegans* L1CAM homologue, *lad-1*/*sax-7* to determine L1CAM functions and mechanisms. Genetic mutants with impaired *lad-1*/*sax-7* function exhibit pleiotropic phenotypes; these include abnormal axon trajectories, neuronal cell body distribution, and gonad morphology. These phenotypes are obvious in adult but not younger animals, suggesting they are a result of defective maintenance of tissue attachment. In addition to these phenotypes, *lad-1*/*sax-7* mutant animals also show variable embryonic lethality. Analysis of these embryos suggests that *lad-1*/*sax-7* plays a role in both embryonic morphogenesis and muscle function. Interestingly, animals that are genetically null for *lad-1*/*sax-7* exhibit uncoordinated movements as well as aldicarb and levamisole resistance, phenotypes that are not exhibited by *lad-1*/*sax-7* hypomorphs, suggesting that *lad-1/sax-7* may play a role in synaptic function. We will be reporting our results on our analysis of this potential synaptic function for *lad-1*/*sax-7*.

2086

**_Sec5_ Genetics and its Localization and Function at the _Drosophila_ Neuromuscular Junction**

R. O. Teodoro, T. L. Schwarz; Neurology, Childrens Hospital, Harvard Medical School, Boston, MA

Many forms of membrane traffic contribute to the development and function of the nervous system. Moreover, neurons, like most eukaryotic cells, need to control the location of distinct forms of exocytosis in order to form and maintain specialized cellular domains. Localized exocytosis likely helps shape the branching of axons and dendrites and the growth of synaptic boutons. The exocyst is a protein complex that is essential for polarized secretion. In *Drosophila*, mutants in the exocyst protein Sec5 are defective in many trafficking assays, including neurite extension, but do not show any defects in secretion of neurotransmitter. Here, we show that Sec5 is enriched at the neuromuscular junction. Because bouton number is linked to muscle size, we rescued Sec5 expression selectively in either neurons or muscle. Expressing Sec5 in the nervous system does not prevent *sec5* mutants from dying as 1$^{st}$ instar larvae, but it appears to give rise to more elaborate neuromuscular junctions. Muscle rescue of Sec5 prolongs the lifespan of the mutants to the 2$^{nd}$ instar, but the morphology of the junctions is defective. Together, these results suggest that muscle size and bouton number can be uncoupled by selective restoration of Sec5 function. To identify genes that interact with the exocyst, we are conducting an enhancer/suppressor screen using a temperature sensitive (ts) allele of *sec5*. *Sec5$^{ts}$* has a complete eye at 18$^0$C, a partial eye at 21$^0$C, and no eye at 25$^0$C. Thus this genotype at 21$^0$C provides a suitable sensitized background for uncovering enhancers and suppressors of the eye phenotype. The identification of genes in the same genetic pathway as the exocyst will likely offer new insights into the mechanism through which the exocyst acts and how it regulates other pathways.

2087

**Differential Regulation of Peripheral Arborization and Central Afferent Bifurcation of Primary Sensory Neurons by Slit/Robo Signaling**

L. Ma, A. Plump, M. Tessier-Lavgine; Biological Sciences, Stanford University, Stanford, CA

To build functional neural networks, neurons like those in the dorsal root ganglion (DRG) develop unique branched structures for their peripheral projections in the skin and for their central projections in the spinal cord (SC). We report that the formation of these different structures is regulated by the Slit/Robo signaling pathway. First, the secreted molecule Slit2 was shown to stimulate branching formation of dissociated DRG neurons cultured in collagen gels. Using this assay, we demonstrate that this positive activity can be mimicked by activation of the Slit receptor Robos, using either specific antibodies or myristyolated cytoplasmic domains. Furthermore, in mice lacking both *Robo1* and *Robo2* or missing all three Slit genes, we found a reduction in ophthalmic branches of trigeminal sensory neurons, suggesting that Slit/Robo is required for proper peripheral arborization. Second, by studying the central projection of DRG neurons in the SC, we found that the same pathway is also required for proper bifurcation, another branching step important for sensory axons to make proper connections in the SC. In mice lacking either both *Slit1* and *Slit2* or both *Robo1* and *Robo2*, sensory axons penetrate the spinal cord prematurely and the dorsal root entry site is mislocalized. Interestingly, single cell analysis suggests that this defect is due to the loss of a negative control over neurite growth direction during or after bifurcation. This conclusion is supported by a preliminary finding that the growth cone of young DRG cells can be transiently collapsed by Slit proteins in vitro. Taken together, our study reveals that Slit/Robo signaling contributes to patterning different branching morphologies of sensory neurons and its action, whether positive or negative, depends on the location and the age of these cells.

2088

**LIM Kinase1 Controls Synapse Stability Downstream of the Type II BMP Receptor**

B. A. Eaton, G. W. Davis; Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA

The formation of neural circuitry during development involves exuberant synaptic growth followed by local remodeling events that result in the stabilization of some synaptic contacts and the elimination of others. The molecular mechanisms that specify which synapses will be retained and which will be eliminated remain largely unknown. Here we demonstrate that the BMP receptor *wishful-thinking* (*wit*) is not only required for synaptic growth, as previously demonstrated, but is also necessary for synapse stabilization at the Drosophila NMJ. Mutations that eliminate the Wit receptor or the BMP ligand *glass-bottom-boat* lead to synapse disassembly and presynaptic retraction. Remarkably, analysis of the *mad* and *medea* mutations demonstrates that Smad-mediated signaling, acting downstream of the Wit receptor, is required for synapse growth but is not necessary for synapse stabilization. We identify LIM Kinase (DLIMK1)-dependent signaling as a second, parallel pathway that confers the added synapse stabilizing activity of the Wit receptor. We show that DLIMK1 binds a region of the Wit receptor that is necessary for synaptic stability but is dispensable for Smad-mediated synaptic growth. A genetic analysis demonstrates that DLIMK1 is necessary, presynaptically, for synapse stabilization but is not necessary for normal synaptic growth or function. Furthermore, presynaptic expression of DLIMK1 in a *wit* or *mad* mutant significantly rescues synaptic stability, growth, and function. DLIMK1 localizes near synaptic microtubules and functions independent of ADF/cofilin, highlighting a novel requirement for DLIMK1 during synapse stabilization rather than actin-dependent axon outgrowth. These data support a model in which non-canonical signaling downstream of the BMP receptor to DLIMK1 is necessary for presynaptic stabilization in response to postsynaptic trophic support.

2089

**Neuronal Growth on a Chip: Integration of Multiple Guidance Cues**

N. Li, T. M. Keenan, C. Hsu, A. Folch; Bioengineering, University of Washington, Seattle, WA

During embryonic neural development, axon tips (“growth cones”) are guided through a dynamic three-dimensional (3-D) landscape by soluble chemotropic factors and by immobilized, growth-permissive or growth-inhibiting contact cues present in the extracellular matrix and on the surface of surrounding cells. It has been difficult to probe the search algorithms of growth cones in response to multiple guidance cues during 3-D navigation due to the limitations of traditional cell culture methods. Our goal is to use micro/nano-fabrication techniques to grow murine embryonic cortical neurons in a microfluidic chip and to present the axons with multiple competing growth options. As 3-D substrates that feature variations in permissiveness and microtopography, we used poly-D-lysine (PDL) coatings on microfabricated steps of polydimethylsiloxane (PDMS) and complementary features of Matrigel. We found that axons display a preference for PDL over Matrigel and for the straightest path within a distance consistent with the exploratory range of the growth cone. When these two preferences are in conflict, axons choose to grow straight into Matrigel; when the straight path is not permissive, the axon turns in the direction that minimizes the turning angle. Neurons can also be

challenged by multiple gradients of diffusive factors delivered using microfluidic devices. A high-throughput analysis tool was developed to automatically analyze growth dynamics of multiple neurons. Our results suggest that growth cones make navigation decisions by integrating biochemical and topographical cues. The microchip also provides a valuable tool for other basic cell biology studies such as cell polarity, cell migration and chemotaxis.

2090

**Neuronal Cell Culture Matrix for Better Maintainence and Surival of Neuronal Cell Cultures in Tissue Culture**

J. Sharma, D. R. Aguirre, N. DiMassa, H. Eran, R. Perez; Molecular Biology, Celprogen, San Pedro, CA

At present we have studied and developed a novel neuronal cell extracellular matrix which allows the following neuronal cell cultures : hippocampal, cerebral cortex and dentate gyrus to grow and make connections with other neurons in cell culture. The current neuronal cell culture extracellular matrix increases the neuronal surival rates by 85% when compared to poly-lysine D and Laminin coated flasks. The neuronal cell extracellular matrix allows one to subclone individual neurons with their axonal and dendritic connections in tissue cell culture platform. The neuronal cell cultures in this matrix demonstrated 90% pure neuronal cell population with only 5% - 10% astrocyte population in culture prior to the first passage and after the first passage neuronal cell culture populations were maintained at 95% neurons with 2 - 5% astrocytes in cell culture. The neuronal cell population demonstrated the following markers up to 6 passages Neurofilament, and MAP-2. The neuronal cell cultures showed the presence of MBP a marker of Oligodendrocytes in cell culture and were selectively removed from the neuronal cell culture to obtain 90% neuronal cell density in culture in Celprogen's Neuronal Growth media. In conclusion the present study demonstrated that primary neuronal cells can be maintained in tissue culture for 6 passages with better surival rates and showed the presence of neuronal markers. At present with this model system we are in the process of measuring neuronal transmitters and the synaptic proteins to validate the formation of synaptic vessicles in tissue culture model. With the current neuronal extracellular matrix technology which includes Celprogen's neuronal growth media continously circulating 10 ml tissue culture vessel would enable greater succes in the research and development of neuronal transplants for spinal cord injuries.

2091

**An Investigation of the Function of the Stonin 2 Protein in Rat PC12 Cells**

A. A. Wall, L. Kelly; Genetics, University Of Melbourne, Parkville, Australia

The mammalian Stonin proteins are homologues of *Drosophila melanogaster* Stoned B adaptor protein, which is involved in synaptic vesicle recycling. Mammalian Stonin 2 protein contains an N-terminal proline-rich domain that interacts with the clathrin adaptor AP2, the stonin-homology domain and the C-terminal µ-homology domain that has sequence homology to the µ2 subunit of AP2. Stonin 2 has been shown to interact with synaptotagmin I (sytI) and is enriched in the hippocampus suggesting that Stonin 2 has maintained some functional similarity to its *Drosophila* counterpart. Two independent studies of Stonin 2 using independently generated anti-N-terminal antibodies, identified proteins of conflicting size on SDS-PAGE (80kDa (expected size) and 120kDa). We have raised anti-Stonin 2 C-terminal antibodies in sheep, and have characterised endogenous Stonin 2 protein expression in the rat PC12 cell line. RT-PCR has confirmed that stonin 2 is transcribed in PC12 cells. When blots were probed with affinity purified anti-Stonin 2 antibodies only protein species of ~65kDa were observed. When HEK-293T cells, which show no stonin-2 expression, were transfected with a tetra-cysteine tagged full-length Stonin 2 cDNA, the antibodies detected a full-length protein at 120kDa along with 80 kDa and 65kDa breakdown products. This indicates that 65kDa protein seen in PC12 cells is likely to be a proteolytic product of the full length Stonin 2. Immunocytochemistry shows co-localisation of stonin 2 with synaptotagmin I, and sucrose gradients and sub-cellular fractionations of PC12 cells showed that the 65kDa stonin 2 proteolytic products are associated with both synaptic and dense-core vesicle fractions. A stably transfected Stonin 2 PC12 cell-line that has been found to express <50% of the endogenous level of stonin 2 will be used for further functional analysis of the role of stonin 2 *in-vivo*.

2092

**NAC1 is a Potential Substrate Adaptor for Cullin3-based Ubiquitin Ligase (E3) Complex**

H. Shen,[1] N. Champtiaux,[1] L. Korutla,[2] S. Toda,[1] S. A. Mackler,[2] P. W. Kalivas[1]; [1]Dept. of Neuroscience, Medical University of South Carolina, Charleston, SC, [2]Dept. of Medicine and Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA

NAc1, which was up-regulated after chronic cocaine in nucleus accumbens selectively, is a nuclear BTB-POZ protein. Although it can repress reporter gene expression in an *in vitro* transcription assay, we found NAC1 interacted with cullin3 (CUL3) directly through its BTB domain using yeast two-hybrid and GST pulldown assay. Overexpression of AAV-NAC1 in rat primary neurons induced an ubiquitinated inclusion appearing in nucleus, which accumulated CUL-3 and 20S proteasome. Accumulation of the 20s proteasome was by virtue of NAC1 binding to mov34, a subunit of the 26s. The proteasome inhibitor MG132 or PKC activation induced NAC1 translocation to the cytoplasm with 20S proteasome, indicating NAC1 might direct the ubiquitination and degradation of specific cytoplasmic targets. These results reveal NAC1 is a potential substrate adaptor for cullin3-based ubiquitin ligase (E3) complex, which links E3 ligase to the proteasome and thereby accelerates protein degradation via ubiquitination pathway.

2093

**Rescue of the Alpha-syntrophin Knockout Phenotype in Skeletal Muscle by Beta 1- and Beta 2-syntrophins**

R. Sealock, N. R. Kramarcy; Cell & Molecular Physiology, University of North Carolina, Chapel Hill, NC

In α-Syn-/- skeletal muscle, deficiencies include neuronal nitric oxide synthase (nNOS) and aquaporin-4 (AP4) at the sarcolemma and acetylcholine receptor, acetylcholinesterase, and utrophin at the neuromuscular junction (NMJ). The NMJ also shows morphological derangements. These characteristics first appear at day 3 postnatal and progress in spite of the normal presence of β1-syntrophin in neonatal (sternomastoid, gastrocnemius) and adult muscle (gastrocnemius). β2-Syntrophin is also present at the adult NMJ. To test whether these α-syntrophin homologs are intrinsically incapable of compensating for the loss of α-syntrophin, we transgenically overexpressed the β-syntrophins in α-Syn-/- mice under the control of the human skeletal actin promoter. Mice transgenically overexpressing α-syntrophin (from Drs. M. E. Adams and S. C. Froehner; creatine kinase promoter) served as control for overexpression artifacts. We achieved high, uniform levels of expression of each β-syntrophin on the sarcolemma and at the NMJ (similar to the α-syntrophin overexpresser, 10-20-fold total overexpression). Both β-syntrophins restored nNOS,

AP4, AChR and utrophin to wild type or near wild type levels. Morphologically, the fingers of AChR which project beyond synaptic gutters in α-Syn-/- NMJs were rarely present, and the lectin VVA-B4, a marker of junctional folds, labels at wild-type levels. Electron microscopy also shows extensive fold structures, but they are disordered in the β-transgenics: quantitatively, the number of fold openings/micrometer of synaptic cleft was restored to only 52% of wild type by the β2 transgene and 56% by the β1 transgene (compared to 35% in ASyn-/- mice and 80% in α-syntrophin transgenics). We conclude that the β-syntrophins are quantitatively weak in their ability to compensate for α-syntrophin but at non-physiologically high concentrations, they achieve nearly complete compensation in most parameters. Supported by MDA, NINDS.

2094

### Vps33a Regulates Behavior and Cerebellar Purkinje Cell Number

S. Chintala,[1] E. K. Novak,[1] R. Gautam,[1] G. Torres,[2] S. Patel,[2] K. Busch,[2] B. A. Meeder,[1] J. M. Horowitz,[1,3] R. T. Swank[1]; [1]Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY, [2]New York College of Osteopathic Medicine of New York Institute of Technology, Old Westbury, NY, [3]Medaille College, Buffalo, NY

A mutation in Vps33a causes symptoms of Hermansky-Pudlak Syndrome (HPS) in the buff (*bf*) mouse coat color mutant. Vps33a is a member of the Sec1 family and Class C protein complex, which are important in vacuolar biogenesis and protein trafficking from the Golgi to the yeast vacuole. Buff mice and other murine animal models of HPS exhibit defects in intracellular vesicle trafficking, ultimately causing significant abnormalities of lysosome-related organelles such as melanosomes, platelet dense granules and lung lamellar bodies. Since Sec1 family members have been implicated in synaptic transmission, buff mutants were tested for neurological abnormalities. Application of standardized behavioral tests (SHIRPA protocols) demonstrated significant motor deficits in buff mutants, and their severity increased in older (1 year) mutants. These included grip strength, righting reflex and touch escape, and older mice showed severe gait abnormalities. Stick models of behaviour showed a characteristic curving behaviour unlike the translational motion of control mice. Enumeration of Purkinje cells with calbindin, a calcium binding protein, revealed fewer Purkinje cells in young (9 week) buff mice compared to control mice, and this reduction in cell number was more pronounced in old (14 month) mutants. The size of the cerebellum was decreased in mutant compared to control. Also, Glial Fibrillar Acidic Protein (GFAP), concentrated in astrocytes, was upregulated in buff brain. Thus, the buff mouse may be a useful model to investigate the role of vesicle trafficking in Purkinje cell development and maintenance. Likewise, these and related observations suggest that certain types of HPS patients may exhibit neurological abnormalities.

2095

### Functional Study of the Stress Inducible Protein 1: A Cellular Prion Protein Partner

M. H. Lopes,[1] G. N. M. Hajj,[1] A. G. Muras,[1] G. L. Mancini,[1] R. M. S. Castro,[1] K. C. B. Ribeiro,[2] R. R. Brentani,[1] R. Linden,[3] V. R. Martins[1]; [1]Laboratory of Cellular and Molecular Biology, Ludwig Institute for Cancer Research, São Paulo, Brazil, [2]Estatística, Centro de Tratamento e Pesquisa - Hospital do Cancer, São Paulo, Brazil, [3]Instituto de Biofísica, Universidade Federal do Rio de Janeiro, São Paulo, Brazil

Investigation of cellular prion protein ($PrP^c$)-interacting partners is an important tool to understanding not only cellular signaling triggered by, but also the biological function(s) related to $PrP^c$. Accordingly, we have demonstrated that $PrP^c$ interacts with STI1 (Stress inducible protein 1), a co-chaperone protein that modulates Hsp70 and Hsp90 activities. Since, STI1 expression is unknown and $PrP^c$ protein expression outside the nervous system is not well documented, we evaluated the distribution of both proteins in mice embryos at different stages of development. STI1 and $PrP^c$ are abundantly co-expressed in most tissues analyzed, since embryonic-day 10. In addition, we examined the signaling pathways and functional consequences of the $PrP^c$ interaction with STI1 in hippocampal neurons. Both $PrP^c$ and STI1 are highly co-localized in the hippocampus *in situ* and dissociated neurons, indicating that they can interact *in vivo*. STI1 elicited neuritogenesis in wild-type neurons, but not in $PrP^c$-null cells. $PrP^c$-STI1 interaction induced phosphorylation/activation of the mitogen-activated protein (MAP) kinase, which was essential for STI1-promoted neuritogenesis. STI1 also prevented cell death via PKA activation. These results demonstrate that two parallel effects of the $PrP^c$-STI1 interaction, neuritogenesis and neuroprotection, are mediated by distinct signaling pathways. Supported by FAPESP.

## Endosomes & Lysosomes I (2096-2108)

2096

### The YXXL Cytoplasmic Motif of HLA-DM Prevents Sorting into Exosomes

A. Brunet,[1] M. Gauvreau,[1] A. Shaw,[2] J. Thibodeau[1]; [1]Microbiology and Immunology, University of Montreal, Montreal, PQ, Canada, [2]Oncology, University of Alberta, Edmonton, AB, Canada

HLA-DM is a non-classical MHC class II molecule that modulates the loading of antigenic peptides on molecules such as HLA-DR. Peptide exchange occurs in class II-rich compartments of the endocytic pathway where DM accumulates due to its tyrosine-based targeting signal. These compartments include multivesicular bodies in which HLA-DR and -DM interact at the level of the internal membranes. Mature peptide-loaded HLA-DR molecules end-up at the cell surface but also accumulate in exosomal vesicles. These small structures are shed presumably through the fusion of multivesicular bodies with the plasma membrane and are obtained by high speed centrifugation of the culture medium. Despite their co-localization with HLA-DR in MHC II-rich compartments, the presence of HLA-DM in exosomes remains to be demonstrated. Here, we show by immunoblot that DM is present in exosomal fractions of the 721.45 B lymphoblastoid cell line. A similar conclusion was reached when looking at exosomes from HeLa cells transfected with CIITA. To gain insight into the intracellular mechanisms regulating exosome entry and cargo retention, HLA-DM was transfected into HeLa cells. Interestingly, in these conditions, DM was excluded from exosomes. However, it was found in exosomes when its YXXL sorting motif was inactivated by site-directed mutagenesis or following co-expression of HLA-DR. Altogether, these results demonstrate that sorting signals in the cytoplasmic domains of transmembrane proteins can regulate exosome entry and raise the intriguing possibility that protein-protein interactions can modulate the activity of such signals.

2097

### Identifying Components of the Adaptor Protein 3 Complex Pathway

V. Anand, G. S. Payne; Biological Chemistry, David Geffen School of Medicine at UCLA, Los Angeles, CA

Eukaryotic organisms distribute specific cellular tasks to specialized membrane-bound organelles. In order to maintain specialization, the subset of proteins required for the organellar tasks must be correctly localized. The adaptor protein 3 complex (AP-3) mediates protein traffic from the Golgi complex to the lysosome in both yeast and mammals. Mutations in human AP-3 can cause the rare heritable genetic disease Hermansky-Pudlak syndrome, a disease characterized by albinism and prolonged bleeding times. In yeast, one AP-3-dependent cargo is alkaline phosphatase (ALP), a zymogen that requires AP-3 mediated transport for efficient maturation. As a systematic approach to identify genes involved in the AP-3 pathway, we have generated whole cell extracts of a *Saccharomyces cerevisiae* single gene deletion library and immunoblotted for the presence of a precursor form of ALP. Preliminary data have identified genes with previously demonstrated involvement in AP-3 mediated transport and those whose specific involvement remains to be determined. One set of genes identified were certain subunits of the v-type vacuolar $H^+$-ATPase. The presence of every subunit of the $H^+$-ATPase is required for its generation of an electro-chemical potential. Therefore the presence of an ALP processing defect in some but not all of the $H^+$-ATPase subunits indicates that its function in the AP-3 pathway is independent of its electro-chemical generating potential.

2098

**Novel Chemical Inhibitors of Membrane Traffic Identified by Chemical-genetic Synthetic Lethal Screening**

M. C. Duncan,[1] D. G. Ho,[2] M. E. Jung,[2] G. S. Payne[1]; [1]Biological Chemistry, UCLA-Medical School, Los Angeles, CA, [2]Chemistry and Biochemistry, UCLA-Medical School, Los Angeles, CA

Small molecule inhibitors have proven to be powerful tools in cell biology. Here we describe a simple, high throughput method for chemical inhibitor identification that we have used to identify novel inhibitors of clathrin dependant traffic. The technique is based on synthetic genetic lethality - severe growth defects caused by specific combinations of mutations - but in this case the combination consists of mutation and chemical inhibitor. The strategy involves a facile and rapid growth-based screen for chemicals that recapitulate known synthetic genetic growth defects when applied to a particular mutant strain. We applied this technique to identify specific inhibitors of clathrin adaptor AP-1-dependant traffic between the TGN and endosomes. We screened ~30,000 chemicals and obtained three inhibitors. These inhibitors, which share a piperazinyl-phenyl-ethanone backbone, cause effects that mimic phenotypes of deletions of clathrin adaptor AP-1 subunits. For example, the compounds alter trafficking of AP-1 dependant cargoes, but do not alter endocytic trafficking. Additionally, these compounds cause synthetic growth defects similar to deletions of AP-1 when combined with different mutations. Limited structure-activity relationship analysis has been performed to identify key characteristics of active compounds. These novel compounds should be useful for further studies of membrane traffic in yeast, and potentially in other systems. Our results establish the efficacy of chemical-genetic synthetic lethal screening, a procedure that may be generally applicable for identification of small molecule inhibitors in a wide variety of other biological processes.

2099

**Vacuolar Membrane Deterioration Leads to Aging**

F. Tang; Biology, University of Arkansas at Little Rock, Little Rock, AR

In *Saccharomyces cerevisiae*, vacuoles degrade damaged proteins and organelles. Malfunction of this vacuole-mediated degradation may accumulate damaged proteins and organelles and thus lead to cell aging. Indeed, autophagy, one vacuolar degradation pathway, is required for life span extension in *daf-2* mutant of *C. elegans*. In this study, we analyzed the roles of vacuoles in the life span extension of yeast. A well established life span-extension treatment is caloric restriction. When yeast cells were cultivated in caloric restricted (low glucose) media, the autophagic activity was up-regulated. Moreover, caloric restriction induced vacuole inheritance defect; more than 50% of cells trapped vacuoles in the mother cell (vacuole inheritance defect) in low glucose medium, whereas only less than 10% of cells show the defect in media with normal glucose concentration. These observations indicate that vacuole inheritance may shorten the life span of the mother cell. One method to measure the life span of yeast is to count the number of buds produced by a mother cell in her whole life. A comparison of the life span of vacuole inheritance mutants indicated that blocking vacuole inheritance (*vac17*Δ) extends life span while accelerating the vacuole inheritance (VAC17-ΔPEST) shortens the life span. The transport complex (Myo2p-Vac17p-Vac8p) for vacuole movement localizes on selected microdomains on the vacuole membrane. These microdomains are most likely ordered microdomains. Blocking the assembly of ordered microdomain by *erg6Δ* or *sur4Δ* cause vacuole inheritance defect and shorten life span, implying that both vacuole inheritance and life span extension require the ordered microdomain. We propose that vacuole inheritance causes a deficit of ordered microdomains. This deterioration decreases the autophagic activity and shortens the life span.

2100

**Vta1 Stimulates the AAA-ATPAse Vps4**

I. F. Azmi,[1] B. Davies,[1] C. Dimaano,[2] P. Johanna,[1] M. Babst,[2] D. J. Katzmann[1]; [1]Biochemistry and Molecular Biology, Mayo Clinic College of medicine, Rochester, MN, [2]Department of Biology, University of Utah, Salt Lake City, UT

Down-regulation of activated receptors is crucial to prevent aberrant downstream signaling which can contribute to uncontrolled cellular proliferation. Internalized activated receptors enter multivesicular bodies (MVB) en route to degradation in the vacuole. Hence in eukaryotes, the MVB pathway plays an essential role in regulating cell surface protein composition and therefore is important for numerous cellular functions. Recent characterization of the class E Vps or Endosomal Sorting Complex Required for Transport (ESCRT) proteins has provided insights into the intricate mechanism governing MVB sorting and led to a working hypothesis for the role of these factors in the MVB sorting pathway. Currently, little is known about regulatory components of the MVB sorting machinery. The class E protein Vps4, an AAA-ATPase, acts late in the MVB sorting reaction to dissociate the ESCRT components and facilitate continued sorting. However, the regulation of Vps4 is not well defined to date. By utilizing biochemical and microscopic analysis in *Saccharomyces cerevisiae* we have characterized Vta1 as a positive modulator of Vps4 both *in vivo* and *in vitro*. Vta1 stimulates the ATPase activity of Vps4 through promoting or stabilizing Vps4 ologomerization. Furthermore we demonstrate that an evolutionarily conserved domain is necessary and partially sufficient for Vps4 binding and stimulation. We conclude that Vta1 homologs regulate Vps4-dependendant disassembly of ESCRT proteins through an evolutionarily conserved mechanism.

2101

### *env1* Allele of *VPS35* Suggests Additional Function for the Gene Product in *Saccharomyces cerevisiae*

C. Frost,[1] M. K. Takahashi,[2] E. Gharakhanian[3]; [1]Department of Chemistry and Biochemistry, California State University, Long Beach, Long Beach, CA, [2]Department of Biological Sciences, California State University, Long Beach, Long Beach, CA, [3]Biological Sciences, California State University, Long Beach, Long Beach, CA

Recognition, sorting, and localization of proteases to the degradative lysosome are vital for the operation of eukaryotic cells. We have utilized a novel mutant screen to isolate mutants defective in trafficking events at the endosome-to-lysosomal vacuole interface in the yeast *Saccharomyces cerevisiae* -- *env mutants. env1* was identified as an allele of *VPS35*. *env1* cells were compared to other *VPS35* allelic and deletion mutants for growth characteristics, Carboxypeptidase Y localization and processing, and vacuolar morphology. The *env1* mutant demonstrates pleiotrophic phenotypes that differ from those previously characterized for *vps35* mutants. e*nv1* also shows intragenic complementation with the *vps35-10* allele. Vps35p has been identified as a member of the protein complex involved in retrograde transport from the late endosome to TGN. Our results suggest an additional role for Vps35p at the late endosome-to-lysosome interface.

2102

### Structure and Function of Nucleus-Vacuole Junctions in *Saccharomyces cerevisiae*

E. L. Kvam, D. S. Goldfarb; Biology, University of Rochester, Rochester, NY

Nonessential portions of the *Saccharomyces cerevisiae* nucleus are targeted to the vacuole and degraded by “piecemeal microautophagy of the nucleus” (PMN). During PMN, small teardrop-like nuclear blebs are engulfed by invaginations of the vacuole membrane, pinched into the vacuole lumen, and degraded by lumenal hydrolases. PMN occurs in the context of nucleus-vacuole (NV) junctions, which are Velcro-like patches formed through specific interactions between Vac8p on the vacuole membrane and Nvj1p in the outer nuclear membrane (perinuclear ER). PMN occurs at low levels in log phase cells, but is induced by nutrient limitation. Although several other ER-organelle membrane contact sites have been observed in eukaryotes, NV junctions are unique because the bridging apparatus that links the perinuclear ER to the vacuole has been identified. Nvj1p binds and sequesters several proteins, including Vac8p, Tsc13p, and Osh1p, to the outer nuclear envelope, thereby creating a unique subdomain in the perinuclear ER. Mutations that affect the activity of Tsc13p, which is required for the biosynthesis of very long chain fatty acids, reduce the size and volume of PMN structures. PMN is also affected by mutations in the *OSH* family of genes, which are thought to mediate non-vesicular lipid transport. These results suggest that the lipid composition of NV junctions regulates the formation of PMN structures. NV junctions may also have roles in cellular lipid homeostasis. We have mapped the three partner-binding domains of Nvj1p and determined that its unusual N-terminal domain is important for targeting to the perinuclear ER. These four domains represent the major regions of sequence conservation among *NVJ1* homologs in several divergent yeasts. NV junctions represent a unique model system for studying the biology of ER membrane contact sites and, as well, the molecular mechanism of selective microautophagy.

2103

### Coordination of Membrane Dynamics by Dynamin and SNARE Proteins

T. L. Baars, A. Mayer; Department of Biochemistry, University of Lausanne, Epalinges, Switzerland

Vesicular traffic involves membrane fission and fusion, two antagonistic processes which need to be carefully balanced to ensure transport between compartments while maintaining organelle identity. Whereas the molecular machinery mediating fission and fusion has been studied in detail only little is known about factors coordinating these membrane rearrangements. The vacuole of the budding yeast *Saccharomyces cerevisiae* has served as a model for studying membrane dynamics. Vacuoles undergo regulated cycles of membrane fission and fusion in the course of the cell cycle and in adaptation to changing environmental conditions. To gain insight into the coordination of vacuolar membrane remodelling we microscopically screened a set of yeast mutants for aberrant vacuolar morphology and disturbed response to osmotic stress. Our data reveal a role of the vacuolar-ATPase in stress-induced fragmentation and suggest that an acidification-defect-associated phenotype may be epistatic to vacuole fragmentation. Moreover the study shows the involvement of the yeast dynamin-like protein Vps1p and of SNARE proteins in maintenance of the equilibrium between membrane fission and fusion. We propose that an interplay between dynamin and SNARE proteins may represent a general concept for regulation of membrane dynamics.

2104

### A TGN Golgin Required for Mannose 6-Phosphate Receptor Recycling

J. V. Reddy, A. Schweizer Burguete, I. G. Ganley, S. R. Pfeffer; Department of Biochemistry, Stanford University School of Medicine, Stanford, CA

Mannose 6-phosphate receptors (MPRs) transport lysosomal enzymes from the trans-Golgi network (TGN) to endosomes. MPRs recycle back to the Golgi where they can participate in further rounds of transport. MPR recycling requires both the Rab9 GTPase and the cytosolic adaptor protein, TIP47. Rab9 positive transport vesicles have been observed to fuse directly with the TGN, yet nothing is known about how these transport vesicles dock with their target membrane. We show here that a member of the Golgin family of TGN localized proteins, GCC185, is a novel Rab9 effector. GCC185 binds Rab9 GTP in preference to Rab9 GDP and fails to bind Rab5. A tyrosine residue that is key for Arl1 binding to this family of TGN Golgins is not needed for Rab9 interaction. GCC185 is required for MPR recycling from endosomes to the TGN in living cells, as monitored visually by light microscopy and also biochemically, using an assay that monitors the tyrosine sulfation of a modified, cation-dependent MPR. A soluble fragment encoding the Rab9 binding site of GCC185 also inhibits MPR recycling in vitro. GCC185 does not rely on Rab9 for its TGN localization; depletion of GCC185 alters the Golgi ribbon slightly but does not interfere with Golgi function. Together, these data suggest that GCC185 is a key component involved in the tethering and docking of Rab9-bearing transport vesicles at the TGN.

2105

### CD1a Co-Localizes with MHC Class I in the Rab22a-Dependent Recycling Pathway

D. C. Barral, M. B. Brenner; Rheumatology, Immunology and Allergy, Brigham and Women's Hospital / Harvard Medical School, Boston, MA

CD1 proteins are major histocompatibility complex (MHC)-like antigen-presenting molecules that present lipid and glycolipid antigens to T cells.

The trafficking of the different CD1 molecules (a, b, c and d) has been shown to be clearly distinct. The intracellular localization of CD1 proteins is thought to intersect the compartments where the antigens traffic to and this could be the basis for the different existing trafficking pathways. After internalization, CD1b traffics deeply into the endocytic pathway and co-localizes almost exclusively with markers of late endosomes and lysosomes. On the contrary, CD1a does not traffic to late endocytic compartments and recycles back to the plasma membrane. In this study we sought to characterize the intracellular pathways followed by CD1a. By confocal microscopy we showed that CD1a co-localizes to a great extent with MHC class I (MHC I) in early/recycling endosomes of HeLa cells, identified by the presence of internalized transferrin ligand. Recently, MHC I was found to follow a Rab22a-dependent recycling pathway, which is used by cargo internalized independently of clathrin. Interestingly, CD1a co-localizes with the small GTPase Rab22a in the recycling tubular structures that contain cargo internalized independently of clathrin, such as MHC I. Moreover, CD1a, but not transferrin, accumulates with MHC I in the enlarged vesicles formed when cells are transfected with the Arf6 Q67L constitutively active mutant, suggesting that CD1a follows an Arf6-dependent endosomal recycling pathway. These findings shed light on the intracellular pathways followed by CD1a and suggest that CD1a is recycled with cargo that shows clathrin-independent internalization. Furthermore, these results link CD1a to the clathrin-independent internalization pathway, contrasting with the other CD1 isoforms, which are internalized in a clathrin-dependent process.

2106

### Rip11/Rab11 Interaction with Kinesin II Regulates the Sorting of Membrane Proteins in the Recycling Endosomes

G. Wilson,[1] K. Anderson,[1] E. Schonteich,[1] J. Burden,[2] C. Hopkins,[2] R. Prekeris[1]; [1]Cellular and Developmental Biology, UCHSC, Aurora, CO, [2]Imperial College, London, United Kingdom

Rip11 is a Rab11-interacting protein that belongs to a FIP protein family. We have previously shown (Peden et al., 2004; also see Burden et al. poster) that Rip11 is localized to recycling endosomes, where it regulates membrane protein sorting to a degradative pathway. In this study we identify kinesin II as a Rip11-binding protein that also plays an important role in protein sorting. Using yeast-two hybrid and a bead pull-down assays we have show that Rip11 and kinesin II interaction is independent of Rab11 binding and mapped the Rip11-binding region to a tail domain of Kif3B and/or Kif3A subunits of kinesin II. Furthermore, we used microtubule binding assays to show that Rip11 binds to microtubules by interacting with kinesin II, and that Rip11 binding to mirotubules can be blocked by 5 mM ATP or Kif3B tail domain. We have also shown that both Rip11 and kinesin II are enriched in purified recycling endosomes. Finally, we have used RNA interference analysis to show that both Rip11 and kinesin II are required for the sorting of CD63 and Lamp1 to the lysosomes while having little effect on transferrin receptor recycling.

2107

### Rab7 Regulation of EGFR Endocytic Trafficking

B. P. Ceresa; Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

The coordinated activation and degradation of the EGFR is a complex process that is regulated at many levels. Ligand binding to the EGFR triggers internalization of the ligand:receptor complex through a clathrin-mediated pathway. The complex is subsequently delivered to the early endosome, late endosome, and lysosome for degradation. Each step of endocytic trafficking is regulated by a unique set of proteins. Through the use of activating and inactivating mutants, we have identified the small molecular weight guanine nucleotide binding protein (G-protein) rab7 as regulator of EGFR trafficking between the late endosome and lysosome. Expression of the activating mutant rab7(Q67L), accelerates the rate of radiolabeled EGF degradation; expression of dominant negative rab7 [rab7(Q67L)] slows the rate of 125I-EGF and EGFR degradation. The change in rates of degradation are due to a defect in endocytic trafficking as rab7(N125I) expression inhibits the migration of the 125I-EGF from light to heavy endosome as determined by migration of Percoll gradients. These experiments reveal that rab7(N125I) causes an accumulation of 125I-EGF in an endosomal compartment of the same density of the late endosome. Therefore, we conclude that rab7 activity is necessary for efficient trafficking of the EGFR to the lysosome.

2108

### Regulation of E-cadherin Trafficking during Epithelial Cell Scattering

J. S. Tushir, F. Palacios, C. D'Souza-Schorey; Biological Sciences, University of Notre Dame, Notre Dame, IN

The constitutive endocytosis and recycling of E-cadherin constitutes a required step in maintaining the dynamics of the epithelial monolayer. Alterations in E-cadherin traffic or a decrease in the surface levels of the E-cadherin complex, results in decreased cell adhesion and in some instances in epithelial to mesenchymal transitions. We have shown that upon v-Src activation, a cell signal that initiates cell scattering during epithelial to mesenchymal transitions, E-cadherin is ubiquitinated and shuttled to the lysosome for degradation, instead of following its normal route of recycling back to the cell surface. Thus Src-regulated lysosomal targeting of E-cadherin constitutes an efficient mechanism to disassemble cell-cell contacts during cell scattering. We are interested in investigating the regulation of E-cadherin traffic to the lysosome. We have found that Src signaling does not result in the activation of metalloproteases; however interference with lysosomal function blocks E-cadherin degradation and epithelial cell scattering. The sorting of ubiquitin-tagged E-cadherin to the lysosome is mediated by Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), an endosomal scaffolding protein, as well the GTPases, Rab5 and Rab7. The interaction of Hrs via its UIM (ubiquitin interaction motif) domain, and its co-localization with ubiquitin-tagged E-cadherin is required for efficient lysosomal targeting of cadherin. However, E-cadherin degradation in lysosomes is independent of Src-induced phosphorylation of Hrs. These investigations demonstrate a pivotal role for endosomal membrane traffic during epithelial cell scattering and describe some key regulators of this process.

## Endothelial Cells (2109-2120)

2109

### Physiologic Cyclic Strain Inhibits the Proliferation of Vascular Endothelium

W. Durante, D. Ensenat, K. J. Peyton; Medicine, Baylor College of Medicine, Houston, TX

Vascular endothelial cells (ECs) are constantly exposed to cyclic strain that arises from the periodic change in vessel wall diameter as a result of pulsatile blood flow. Recent scientific studies indicate that cyclic strain regulates EC orientation, permeability, and apoptosis. In the present study,

we investigated whether cyclic strain also influences EC growth. Cultured human dermal microvascular ECs or bovine aortic ECs were plated onto collagen-coated Bioflex plates and subjected to a physiological level of cyclic strain (6% at 1 hertz) using the Flexercell 3000 Strain Unit. Treatment of human or bovine ECs with fetal bovine serum (5%) for 7 days stimulated a time-dependent increase in cell number that was markedly reduced by the application of cyclic strain. A significant decrease in the number of ECs was first noted after 3 days and this became more pronounced after 7 days of cyclic strain. The cyclic strain-mediated decrease in EC number was not due to cell detachment, necrosis, or apoptosis. Cyclic strain increased the fraction of cells in $G_2M$ and decreased the percentage of cells in the $G_0/G_1$ phase of the cell cycle. Cyclic strain had no effect on the expression of cyclin D1, cyclin E, cyclin A, and cyclin B1, or activation of retinoblastoma protein. In contrast, cyclic strain stimulated the expression of the cyclin-dependent kinase inhibitor, p21. In conclusion, the present study demonstrates that a physiologically relevant level of cyclic strain (6% at 1 hertz) inhibits the proliferation of ECs derived from different species and vessels. This antiproliferative effect is associated with the induction of p21 and the arrest of ECs in the $G_2M$ phase of the cell cycle. The ability of cyclic strain to inhibit EC growth provides an additional mechanism by which hemodynamic forces regulate EC function.

2110

**Norepinephrine Evokes Changes in Aortic Endothelial Cell Morphology Through PKA-mediated Processes**

S. Dissing, S. Ditlev, K. Tritsaris; Department of Medical Physiology, University of Copenhagen, Copenhagen, Denmark

New stable blood vessels are formed through stimulation of endothelial cells by several growth factors such as VEGF, PDGF and FGF. However, other agonists as for instance catecholamines might also play an important role in mediating the formation of organized, functional vessels through cell activations as well as through apoptotic processes. We have studied possible interactions between the sympathetic nervous system and VEGF-mediated controll of angiogenesis using cultured pig aortic endothelial (PAE) cells that express the VEGFR-2 and we found that norepinephrine (NE) stimulation interacts with cellular signaling regulated by VEGF. NE leads, besides $Ca^{2+}$ signaling, activation of Src and formation of NO and cAMP, also to dephosphorylation of the anti-apoptotic signaling enzyme Akt (protein kinase B). The decrease in phosphorylation of Akt is mediated through cAMP, activation of protein kinase A and the Src tyrosin kinase. NE also affects the growth factor-induced tube formation since endothelial cells grown on matrigels in the presence of 10 µM NE appear as small, thin features. Cells display long protrusions often with an unstructured appearance lacking the ability to regulate tube formation. We used small interference RNA directed against PKA to reduce the expression of PKA specifically. This resulted in reduction of the PKA mRNA level by more than 80 % and the cellular amount of PKA by 70 % as evaluated using real time PCR and western blotting. The RNA interference dampened the NE effect on cell structure changes, consistent with PKA activity playing a role in regulation of cell morphology. In conclusion the interplay between sympathetic and growth factor stimulation seems to involve NE-mediated attenuation of Akt activity, resulting in changes of endothelial cell structure that might influence the formation of new blood vessels.

2111

**Embryonic Stem Cell-Derived Endothelial Cells and In Vivo-Derived Endothelial Cells: A Comparison**

K. E. McCloskey,[1,2] D. Smith,[3] H. Song,[4] H. Jo,[4,3] R. Nerem[2]; [1]Bioengineering, UC Merced, Merced, CA, [2]Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, [3]Cardiology, Emory University, Atlanta, GA, [4]Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA

Stem cell research has gained in popularity due to their potential to repopulate specific tissues that may be damaged. Many cell therapies involve in vitro stem cell differentiation, however; tissue-specific cells derived from stem cells in vitro have a very different history compared with cells derived in vivo. Stem cell therapies will only become clinically relevant if the stem cells differentiated in vitro function as their in vivo counterparts. Our laboratory has developed techniques for isolating a highly purified population of endothelial cells (>96% purity) from mouse embryonic stem (ES) cells. ES-derived endothelial cells (ECs) stained positive for EC markers Flk-1, Flt-1, vascular endothelial cadherin (VEcad), platelet-endothelial cell adhesion molecule-1 (PECAM-1), CD105, and CD34. Comparisons of in vitro-derived ECs with endothelial cells isolated from the mouse aorta (MAECs) indicate that both ES-derived ECs and MAECs are approximately the same size and have comparable proliferation rates. They also express similar amounts of endothelial nitric oxide synthase, VE-cadherin, PECAM1, Flk1, and Flt1, however, the EC-derived ECs incorporate lower levels of low density lipoprotein (LDL) and express less von Willebrand Factor than mature normal MAECs. Alternatively, ES-derived ECs exhibit greater angiogenic behavior both in collagen gels and on Matrigel than MAECs, a potential indicator that ES-derived ECs are not completely “mature” cells and, therefore, a potentially ideal cell source for vasculogenic therapies.

2112

**FcγRIIB Mediates C-Reactive Protein Inhibition of Endothelial NO Synthase**

C. Mineo,[1] A. K. Gormley,[1] I. S. Yuhanna,[1] S. Osbourne-Lawrence,[1] L. L. Gibson,[1] L. Hahner,[1] R. V. Shohet,[2] S. Black,[3] J. E. Salmon,[4] D. Samols,[3] D. R. Karp,[2] G. D. Thomas,[2] P. W. Shaul[1]; [1]Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX, [2]Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, [3]Biochemistry, Case Western Reserve University, Cleveland, OH, [4]Medicine, Cornell University Weill Medical College, New York, NY

C-reactive protein (CRP) is an acute phase reactant which is positively correlated with cardiovascular disease risk and endothelial dysfunction. Whether CRP has direct actions on endothelium and the mechanisms underlying such actions are poorly understood. We have demonstrated in cultured endothelial cells that CRP prevents endothelial NO synthase (eNOS) activation by diverse agonists including vascular endothelial growth factor, insulin, high-density lipoprotein and acetylcholine (Ach). Diminished NO production through eNOS inhibition by CRP results in the promotion of monocyte adhesion to endothelial cells. CRP antagonism of eNOS occurs nongenomically and is due to blunted eNOS phosphorylation at Ser-1179. Treatment of endothelial cells with okadaic acid reverses CRP antagonism of eNOS, indicating a key role for the phosphatase,PP2A. Aggregated IgG, the known ligand for Fcγ receptors, causes parallel okadaic acid-sensitive loss of eNOS function, indicating a role for FcγRIIB in CRP- and aggregated IgG-mediated eNOS antagonism. We further showed that FcγRIIB is expressed in endothelium, and that CRP antagonism of eNOS requires FcγRIIB in heterologous expression studies in COS-7 cells. Moreover, in $Fc\gamma RIIB^{+/+}$ mice CRP blunts Ach-induced increase in carotid artery vascular conductance; in contrast, CRP enhances Ach responses in $Fc\gamma RIIB^{-/-}$ mice. These collective findings indicate that FcγRIIB mediates CRP inhibition of eNOS via PP2A, providing a mechanistic link between CRP and endothelial dysfunction.

2113

**Visualization of Morphological Changes of Single Endothelial Cells in Response to Cyclic Stretch**

S. Iwayoshi,[1] K. Furukawa,[1] T. Ushida[2]; [1]Department of Mechanical Engineering, School of Engineering, University of Tokyo, Tokyo, Japan, [2]Center for Disease Biology and Integrative Medicine, School of Medicine, University of Tokyo, Tokyo, Japan

Endothelial cells (ECs) are known to change their shapes aligned perpendicular to the direction of cyclic stretch. However, the mechanisms are still not clear. In this study, we developed a new cell-stretching device which applies uniaxial strech to cells cultured on elastic silicone membrane. Since the membrane is equally stretched from both ends by rotation of oval cams, the center of the membrane remains stationary during the stretch motion, which allows continuous visualization of cyclically stretched/relaxed cells. By using this apparatus, morphological changes of single human vein endothelial ECs in response to cyclic stretch (1Hz, 10%, 2 hours) were visualized and analyzed. The data showed that there were two major patterns of morphological changes of ECs, depending on their initial shapes. While ECs with round shapes at initial state changed their shapes to become more elongated in a direction perpendicular to that of the stretch, cells oriented and elongated in the stretch direction at initial state changed their shapes to become rounder first and then to become elongated in the direction perpendicular to that of the stretch. Cytoskeletal visualization of human aortic ECs with GFP-tagged actin also showed contraction of stress fibers oriented in the stretch direction after 30 min cyclic stretch, which resulted in the orientation of the ECs in the direction perpendicular to that of the stretch. In addition, the cyclic-stretch-induced endothelial morphological change was blocked by Y-27632, a Rho-related kinase inhibitor, or blebbistatin, a myosin inhibitor. Taken together, cell retraction as well as elongation could play important roles in the stretch-induced endothelial morphological changes, and this retraction might be induced by stress fiber contraction via ROCK-mediated myosin phosphorylation.

2114

**Three-Dimensional Visualization of Weibel-Palade Bodies by Electron Tomography**

K. M. Valentijn, K. A. Jansen, W. J. C. Geerts, J. Post, A. J. Verkleij, A. J. Koster; Molecular Cell Biology, University of Utrecht, Faculty of Biology, Utrecht, The Netherlands

Endothelial cells secrete via regulated exocytosis von Willebrand Factor (vWF), which mediates blood clotting in response to vascular injury. VWF is stored by endothelial cells in cigar-shaped organelles, the so-called Weibel-Palade Bodies (WPBs), of 0.1-0.3 by 1-4 μm in size. Electron microscopy studies have shown that the lumen of WPBs displays typical longitudinal striations that appear as tubular structures in cross sections. Interestingly, vWF self assembles into high molecular weight multimers and immunogold microscopy studies suggest that the striations represent vWF multimers. To this date, little is known on the structural characteristics of these tubular striations. Therefore we have initiated a high resolution microscopic study using electron tomography to gain further understanding of the morphology of this specialized organelle. Thick sections (250 nm) of chemically fixed and epon flat-embedded as well as high-pressure frozen and freeze-substituted human umbilical-vein endothelial cells were studied by electron tomography. We determined that the number of tubular striations ranged from 8 to 28 per WPB, and that they were 11.6 ± 1.9 nm (n=51) in diameter. These striations were equally spaced from each other by a distance of 33.6 ± 5.9 nm (n= 111). Longitudinally sectioned WPBs visualized by electron tomography allowed for the tracking of single tubular striations all the way through the WPBs. While most striations ran longitudinally and parallel to each other, a few started to curve towards the extremities of the WPBs. These data suggest that multimers of vWF are highly organized in WPBs via a yet to be identified scaffold. Currently our research focuses on improving our understanding on the organization of the tubules in WPBs with particular interest on how the tubule organization relates to the cigar shape of WPBs and whether the tubules interact with the limiting membrane of WPBs.

2115

**Nitric Oxide Causes Relaxation of an Endothelial Cell Layer by a Adenylate Cyclase - Independent Mechanism**

B. M. Spataro, G. E. Plopper; Biology, Rensselaer Polytechnic Institute, Troy, NY

Using Electric Cell-substrate Impedance Sensing (ECIS) we have found that addition of 0.2 mM NOC-12, a donor of nitric oxide (NO), causes an increase in resistance in lung endothelial cells. Peaks in resistance occur approximately fifteen hours after the addition of NOC-12. Since "classical" NO-cGMP signaling occurs in minutes, and does not affect resistance in our cells, we assume that other signaling pathways are mediating this response. In testicular epithelial cells, nitric oxide activates production of cyclic AMP by adenylate cyclase and subsequent activation of Protein Kinase A (PKA). This cyclic AMP/PKA pathway regulates tight junction dynamics. We hypothesize that this signaling cascade may be involved in the changes in resistance observed in our cells. We found that elevation of cAMP with IBMX, a cAMP phosphodiesterase inhibitor, induced peaks in resistance that differed from those caused by NOC-12. Addition of a PKA inhibitor had no effect on the increase in resistance caused by NOC-12. These results suggest that NO-induced changes in endothelial resistance are independent of the cyclic AMP/PKA pathway in lung endothelial cells.

2116

**15d-$PGJ_2$ Suppressing MMP-2 Expression is Mediated via Nitric Oxide Induction by p38 Signaling Pathway in Endothelial Cells**

H. Chen; Cardiovascular Disease Division, Institute of Biomedical Sciences,Academia Sinica, Taipei, Taiwan Republic of China

15-Deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) derived from arachidonic acid has been suggested to exert anti-inflammatory effects. The intracellular accumulation of 15d-$PGJ_2$ found in the atherosclerotic lesion may play a role during atherogenesis. 15d-$PGJ_2$ has been elicited to increase expression of activating transcription factor 3 (ATF3), induce production of nitric oxide (NO), and inhibit cell migration in various types of cell. However, the detail mechanisms of the affective role of 15d-$PGJ_2$ in ECs are not clear yet. In the present study, ECs treated with 15d-$PGJ_2$ increased ATF3 protein level in a dose- and time-dependent manner. ECs treated with 15d-$PGJ_2$ increased NO production due to the activation of eNOS. The 15d-$PGJ_2$-induced production of NO and ATF3 were inhibited by pre-treating ECs with L-NAME, a NOS blocker. Likewise our previous study demonstrated that NO suppressed Matrix metalloproteinase-2 (MMP-2) via ATF3 induction. MMP-2 mRNA expression and its promoter activity were suppressed after ECs were treated with 15d-$PGJ_2$. In addition 15d-$PGJ_2$-treated ECs showed a suppressed secretion of MMP-2 as a result was revealed by zymographic assay. SB203580, a p38-pathway inhibitor, but not SP600125 and PD98059, JNK and ERK inhibitors, respectively, is involved in 15d-$PGJ_2$-induced expression of ATF3 in ECs. Consistently 15d-$PGJ_2$-induced expression of ATF3 was suppressed after ECs were transfected with a catalytically inactive mutant of p38. Moreover, 15d-$PGJ_2$-induced phosphorylation of eNOS and

production of NO were attenuated by pre-treating ECs with SB203580. Concordantly these results suggest that 15d-$PGJ_2$ activates p38 signaling pathway followed with eNOS activation and the increased NO production inhibits MMP-2 expression via an induction of ATF3. Thus 15d-$PGJ_2$ may play a negative role by inhibiting endothelial migration via the reduction of MMP-2 expression.

2117

**Calmodulin and Regulation of $Ca^{2+}$ Signaling and Barrier Functions of Brain Endothelial Cells**

Y. V. Kim, V. Siddharthan, K. S. Kim; Pediatrics Infectious Diseases, Johns Hopkins University, Baltimore, MD

Human brain endothelial microvascular cells (hBMEC) are major constituent of blood brain barrier (BBB). Many pathological conditions, such as stroke and inflammation, are associated with $Ca^{2+}$-dependent BBB disruption. Small ubiquitous protein calmodulin (CaM) is the key intracellular $Ca^{2+}$ sensor that transfers agonist-induced $Ca^{2+}$ changes to numerous $Ca^{2+}$/CaM-dependent proteins. However, the role of CaM in BBB regulation is not fully understood. In this study we investigated how CaM antagonists (trifluoperazine, W-7 and calmidazolium) affect $Ca^{2+}$ signaling, barrier functions of hBMECand spatial distribution of junctional proteins. Using single-cell, real-time fluorescent microscopy and continuous monitoring of transendothelial electrical resistance (TEER) we found that CaM antagonist induced dose- and time-dependent prolonged $Ca^{2+}{}_{in}$ increase due to $Ca^{2+}$ release from thapsigargin-sensitive $Ca^{2+}$ pool independently on extracellular $Ca^{2+}$. CaM antagonists did not block initial, phospholipase C-dependent, rapid $Ca^{2+}$ rise induced by G-protein coupled receptor agonists, such as thrombin, ATP or histamine. However, CaM antagonists significantly impaired subsequent opening of the store-operated $Ca^{2+}$ channels (SOCCs) according to the known capacitative calcium entry mechanism. Moreover, despite such prolonged $Ca^{2+}{}_{in}$ increase, CaM antagonists did not induce changes of TEER and permeability across the endothelial cell monolayer. The obtained data suggest that activation of CaM and SOCCs are essential steps in BBB dysfunctions and CaM antagonists may be useful as potential drugs during various neurological disorders.

2118

**Early Dynamics of Tyrosine Phosphorylated Proteins in Cyclically Strained Endothelial Cells**

Y. Chiu,[1,2] K. Fujiwara[1]; [1]Cardiovascular Research Institute, University of Rochester, Rochester, NY, [2]Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY

Cyclic strain, one of the mechanical forces associated with blood flow, has been recognized as an important modulator of vascular cell physiology. While many biochemical modifications and chronic cellular effects have been reported in cyclically stretched cells, the early molecular events involving protein phosphorylation, an essential element of signal transduction, remain to be investigated by proteomic approaches. The goal of this study is to identify phosphoproteins whose level of tyrosine phosphorylation is regulated in early phase of acute cyclic strain. Confluent bovine arterial endothelial cells cultured on an elastic silicon membrane were subjected to uni-axial cyclic stretch (25% in length, 0.5Hz) for up to 60 min. The stretched cells were lysed for biochemical analysis or fixed for morphological study. The general level of protein tyrosine phosphorylation was significantly enhanced and peaked at 5 min in response to stretch. In immunoblots using phosphotyrosine-specific antibodies (4G10), we consistently noted 17 bands whose tyrosine phosphorylation levels changed in a stretch-dependent manner. The 125kD and 42kD bands were identified as FAK and ERK2, respectively. These results are consistent with other reports showing that phosphorylation of FAK and ERK is early molecular event in cyclically stretched cells. More notably, several other proteins showed significant transient increase after the stretch onset, particularly those with molecular masses of approximately 130kD, 126kD, 110kD, 90kD, 72kD, and 68kD. Immunoprecipitation with 4G10 was used to purify these phosphoproteins for MS identification. Immunofluorescence labeling revealed peripheral accumulation of pY397-FAK and cell-cell junction recruitment of general phosphotyrosyl proteins within 10min of stretch, while PECAM-1 distribution had no detectable changes. These data demonstrate the early dynamics of mechano-responsive phosphoproteins and support the notion that cell-cell junction is an important site for mechanosignal transduction.

2119

**Molecular Interaction of Protein-Zero Related in Endothelial Cells**

K. Kusano, T. N. Thomas, K. Fujiwara; Cardiovascular Research Institute, University of Rochester Medical Center, Rochester, NY

Protein-zero related (PZR), an IgV-type immunoreceptor with two ITIMs, is expressed in various cells including vascular endothelial cells (Xu et al., ECR272 (2002)) and we have already cloned bovine PZR from bovine aortic endothelial cells (BAECs). PZR specifically binds to SHP-2, an SH2-domain containing tyrosine phosphatase, when the ITIMs are phosphorylated. In ECs, PECAM-1 and Gab1, two other proteins with two ITIMs, are also expressed and they have been shown to also bind to SHP-2 in a tyrosine phosphorylation-dependent manner. Using anti-SHP-2, we indeed co-immunoprecipitated PZR, PECAM-1, and Gab1 from the extract of BAECs treated with pervanadate and found that these three proteins were the major SHP-2 binding proteins in BAECs. These results suggested that SHP-2-dependent signaling is regulated not only by PECAM-1 and Gab1 but also by PZR in ECs. Because the PECAM-1/SHP-2 association is induced in ECs that are challenged with mechanical stimuli such as fluid shear stress and hyperosmotic shock (HOS), we wondered if PZR/SHP-2 interaction was also heightened by HOS. The SHP-2 interaction with PZR was indeed increased in HOS-stimulated BAECs. However, unlike for PECAM-1, the HOS-stimulated SHP-2 binding to PZR was strongly reduced in the presence of NAC, an anti-oxidant, suggesting that tyrosine phosphorylation of PZR and PECAM-1 is regulated differently in ECs. We also observed that PZR is localized to the cell-cell contact sites and intracellular granules in BAECs as well as REN cells (methothelioma cell line) by immunofluorescence analysis. These results also indicate that tyrosine phosphorylation of PZR is regulated differently and may participate in different signaling events.

2120

**Ellagic Acid Inhibits Il-1β-induced Cell Adhesion Molecule Expression in HUVECs**

Y. Yu,[1] Z. Wang,[1] W. Chang,[2] C. Wu[3]; [1]Nutrition, China Medical University, Taichung, Taiwan Republic of China, [2]Sports Medicine, China Medical University, Taichung, Taiwan Republic of China, [3]Biological Science &Technology, China Medical University, Taichung, Taiwan Republic of China

Expression of cell adhesion molecules by the endothelium and the attachment of monocytes to endothelium may play a major role in atherosclerosis. Ellagic acid is a phenolic compound presents in fruits and nuts including raspberries, strawberries, walnuts and grapes. In this

study, we investigated the antioxidative effects of ellagic acid and expression of vascular cell adhesion molecule induced by IL-1β in human umbilical vein endothelial cells (HUVECs). The result indicated that ellagic acid possess antioxidant property. Ellagic acid significantly reduced the binding of human monocytic cell to IL-1β treated HUVECs. The production of reactive oxygen species (ROS) by IL-1β was dose-dependently suppressed by ellagic acid. Supplementation with increasing doses of ellagic acid up to 50 μM was most effective for the inhibition of the expression of VCAM-1 and E-selectin. Furthermore, the inhibition of IL-1β induced monocyte adhesion by ellagic acid was manifested on the suppression of nuclear translocation of NFκB p65 and p50. In conclusion, ellagic acid inhibits IL-1β induced ROS production and the expression of VCAM-1 and E-selectin, resulting in decrease of monocytes adhesion. Thus ellagic acid has anti-inflammatory properties and may play an important role in the prevention of atherosclerosis.

## Cancer III (2121-2142)

2121

### Progression into Cell Cycle and Neuroendocrine-like Morphological Changes are Induced in Lncap Cells in Response to Osteoblast-derived Factors

A. Beckett,[1] B. A. Masri,[2] H. Hare,[1] N. Mawji,[1] N. Blaszczyk,[1] M. D. Sadar[1]; [1]Genome Sciences Center, BC Cancer Agency, Vancouver, BC, Canada, [2]Orthopaedic Surgery, Vancouver General Hospital, Vancouver, BC, Canada

Osseous prostate cancer metastases constitute a major problem in the clinical management of prostate cancer. The skeleton is the most common site of metastasis for men with prostate cancer and these lesions are associated with poor prognosis. Metastasis to the bone can occur only if cancer cells are able to escape the periprostatic space, enter the circulation, adhere to and invade the bone endothelium and ultimately become established in their new microenvironment. A model was utilized whereby prostate cancer cells were cultured in media conditioned with the secretions from osteoblast-like cells obtained from explants of human trabecular bone. This model mimics the clinical scenario for the disease as indicated by increased expression of prostate-specific antigen and enhanced cellular proliferation of prostate cancer cells. Analysis of gene expression using Affymetrix GeneChips and subsequent validation via competitive reverse-transcription PCR identified a number of genes involved in the G2-M cell cycle transition phase that were up-regulated in prostate cancer cells exposed to osteoblast-derived factors. These genes included Cdc2, cyclin B1, cyclin B2, Cdc25C and Plk1. Concurrently, increased cell viability and an increased number of cells in G2-M and S phase of the cell cycle were measured. Neuroendocrine-like morphology, similar what is observed in the development of androgen-independence, developed in prostate cancer cells after treatment with osteoblast-derived factors. Inhibition of the advancement of prostate cancer cells by intervention through the cell cycle could provide a mechanism by which to prevent the establishment of prostate cancer cells in the bone microenvironment. This may be accomplished by targeting the G2-M phase transitional genes shown to be up-regulated in response to the osteoblast-derived factors effectively causing G2 phase cell cycle arrest.

2122

### Expression Profiling of Genes Correlated with Response to 5-Fluorouracil Chemotherapy in Colon Cancer

R. C. Pezo, R. H. Singer; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Using single cell expression profiling we are examining the transcription of genes involved in the sensitivity of colon cancer cells to 5-Fluorouracil (5-FU). Our goal is to develop a set of markers that would be predictive of response to 5-FU-based chemotherapy and to potentially identify novel genes involved in mediating sensitivity to 5-FU. Probes directly labeled with multiple fluorophores for Fluorescence in situ Hybridization (FISH) can simultaneously visualize multiple nascent messenger RNA transcripts. This yields a gene expression profile for each single cell. Application of this technique to clinical samples allows us to preserve tissue architecture while circumventing the issue of tissue heterogeneity. Gene expression profiles can then be applied to tissue in clinical trials and correlated with outcomes. Thus single cell expression profiling of tumors would allow us to identify important subpopulations of cells which may only represent a minor percentage of the sample but may have important phenotypes such as chemosensitivity or metastatic potential. This technique would provide data that would be valuable in correlating the transcriptional profile of cells with tumorigenesis, metastasis, and drug response. Using this information, chemotherapy treatments can be tailored to the individual patient.

2123

### Identification of Neutralizing Antibodies to a G Protein-Coupled Receptor (GPCR) Ligand, Human Gastrin, Using a Flow Cytometry-based Intracellular Calcium Flux Assay

P. Larsen,[1] A. Patel,[1] E. Pongo,[1] D. Bohmann,[1] J. Brink,[1] T. Neben,[1] M. Roell,[1] R. Hansen,[1] S. Grimes,[2] M. Handa[1]; [1]Preclinical Research, XOMA LLC, Berkeley, CA, [2]Aphton Corporation, Philadelphia, PA

Overexpression of the gastric hormone gastrin has been implicated in tumor progression in gastrointestinal cancers, making it a potential target for antibody therapeutics that neutralize its function. Gastrin binds to a G-Protein coupled receptor (GPCR), $CCK_2R$, and triggers intracellular calcium flux through a phospholipase C (PLC)-mediated signaling pathway. Therefore, in order to identify gastrin neutralizing antibodies, we developed a flow cytometry-based assay to measure gastrin-induced calcium flux in a $CCK_2R$ stably transfected cell line using the calcium sensitive fluorescent dyes Fluo-3 AM and Fura Red AM. Due to the rapid response of the target cell line to gastrin stimulation, the Cytek Time Zero system was used for on-line injection of stimulus. Using this assay, a panel of fully human phage display-derived gastrin specific antibody fragments was screened for gastrin neutralization and compared to top mouse hybridoma-derived monoclonal antibodies. Those showing greater than 70% neutralization were reformatted to whole IgG molecules. Reformatted antibodies were retested, and the top candidates analyzed to generate $IC_{50}$ values. The most potent antibody from the calcium flux assay also exhibited the highest affinity as measured by BIAcore®, suggesting a direct correlation between potency and affinity for this target. The top candidates were tested for neutralization of ERK1/2 phosphorylation and showed similar potency profiles as the calcium flux assay, further supporting the capacity of these antibodies to neutralize cell signaling mediated by gastrin. In summary, we have utilized a FACS-based intracellular calcium flux assay to select a potent neutralizing fully human antibody to gastrin derived from phage display technology which is competitive in function to a lead mouse monoclonal antibody derived from hybridomas.

2124

**The Ubiquitination Activity of BRCA1 in Association with BARD1 Regulates Centrosomal Function**

S. Sankaran, J. D. Parvin; Pathology, Brigham and Womens' Hospital, Boston, MA

The breast and ovarian cancer specific tumor suppressor, BRCA1, has multiple functions aimed at maintaining genomic stability in the cell by participating in a variety of cellular processes including transcription, DNA damage repair, and cell cycle progression. We show that BRCA1 and BARD1 localize to centrosomes throughout the cell cycle, and the E3 ubiquitin ligase activity of the heterodimer inhibits centrosome function. Centrosome function was tested in vivo and in vitro in assays that measure microtubule (MT) nucleation. A point mutant of BRCA1 that is specifically deficient in E3 ubiquitin ligase activity not only fails to inhibit centrosome mediated MT nucleation but leads to hyperactive centrosomes at higher concentrations indicating that the mutant had a dominant negative effect in vitro and in vivo. We mapped domains of BRCA1 and BARD1 required to regulate centrosome function. Although carboxy-terminal truncations of each BRCA1 and BARD1 could produce active E3 ubiquitin ligases, only heterodimers of the full length BRCA1 and BARD1 could regulate centrosome function. Results of the in vitro assay were consistent with in vivo findings. The regulation of centrosome function by BRCA1/BARD1 is the first described functional assay to be regulated by BRCA1-dependent ubiquitination. We conclude that the BRCA1-dependent E3 ubiquitin ligase maintains centrosomes in the quiescent state in breast cells, and loss of BRCA1 in the precancerous breast cell leads to centrosomal hypertrophy, a phenotype commonly observed in incipient breast cancer. Dysregulation of centrosomes would then be mutagenic and cause the aneuploidy of breast tumor cells.

2125

**Secreted Caveolin Promotes Tumor Formation of Human Prostate Cancer Cells in Mice**

R. Bartz,[1] J. Zhou,[2] J. Hsieh,[2] W. Li,[1] R. G. W. Anderson,[1] P. Liu[1]; [1]Cell Biology, UT Southwestern Medical Center at Dallas, Dallas, TX, [2]Urology, UT Southwestern Medical Center at Dallas, Dallas, TX

Caveolae are plasma-membrane domains that form omega-shaped invaginations in many cells. One of the structural components of these lipid domains is caveolin-1 (Cav-1). Cav-1 is also found in lipoprotein particles secreted by exocrine cells. Here, we present evidence that secreted Cav-1 has biological activity. LNCaP cells, a human prostate cancer cell line, do not express endogenous Cav-1. When they are transfected with Cav-1, however, they secrete Cav-1 in lipid particles. To investigate the biological function of this secreted particle, we injected 10 nude mice at six sites with Cav-$1^+$ LNCaP cells ($3x10^5$/site). Another 10 mice were similarly injected with Cav-$1^-$ LNCaP cells as a control. All of the animals injected with Cav-1 expressing LNCaP cells displayed a high incidence of solid tumor formation at the site of injection (2-3 tumors/animal in two trials) whereas no solid tumors were observed at sites of Cav-$1^-$ LNCaP cell injection 8 weeks after injection. To see if it was the Cav-1 secreted by expressing cells that influenced tumor formation, one side of each nude mouse (8 mice) was injected at 3 sites with Cav-$1^+$ LNCaP cells and the contralateral side at 3 sites with Cav-$1^-$ LNCaP cells. As expected, Cav-$1^+$LNCaP cells formed solid tumors at most injection sites (1.5/animal). Surprisingly, the non-expresising cells on the contralateral side also formed solid tumors (1/ animal), suggesting that the Cav-1 secreted by expressing cells was affecting tumor growth of non-expressing cells at a distance. Immunohistochemistry did not detect any Cav-$1^+$ LNCaP cells in the induced tumors. Similar observation were made using the PC3 cell prostate tumor cell, which normally expresses Cav-1. These results suggest that the secreted Cav-1 particle has autocrine and endocrine activity *in vivo.*

2126

**Single Antibody Determination of Phospho- and Non-phospho c-Myc in Small Samples with a New Blotless Nano Western System**

D. W. Voehringer,[1] X. Bi,[1] D. Deb-Basu,[2] A. Fan,[2] D. Felsher,[2] J. Ferrante,[1] C. Kirby,[1] R. O'Neill[1]; [1]Cell Biosciences, Palo Alto, CA, [2]Oncology, Stanford University, Stanford, CA

Overexpression of the c-Myc protein is an underlying cause of many cancers. Normal c-Myc protein levels are low and kept in check through short protein half-life. Previous reports have shown that in c-MYC conditional transgenic model systems, returning c-MYC to normal levels leads to sustained tumor regression. Hence, targeting the inactivation of c-Myc may be useful for the treatment of cancer. One possible therapeutic approach may be to target gene products that regulate c-Myc activation. Myc is known to be regulated by several post-transcriptional modifications, but to date there is no method to monitor these changes in a sensitive and quantitative manner. Here we measure, in a conditional c-Myc cell line stimulated to express c-Myc, protein phosphorylation state using the novel method of Blotless Nano Western. The system first physically separates the phospho- and non-phospho forms of c-Myc in a small capillary. After focusing, in-capillary protein immobilization and subsequent probing with an antibody directed against total c-Myc protein allows for high sensitivity detection of c-Myc protein. Phosphorylated and non-phosphorylated species were separated, and detected by the single antibody enabling the determination of the *level* of c-Myc activation in a single sample. We anticipate this will allow repeat sampling of fine needle aspirates from mouse tumors where current methods require multiple mice for a single conventional Western blot assay. Our approach will be useful in examining key signaling in pathways that control c-Myc status and more importantly be useful in the identification and screening of novel drugs that target c-Myc activation for the treatment of cancer.

2127

**Human Survivin Links the Relocation and Function of Aurora B Kinase to Central Spindle for Completion of Cytokinesis**

Y. Ke,[1] Y. Miao,[1] L. Zhao,[1] H. Deng,[1] F. Wang,[1] Z. Dou,[2] A. Shaw,[2] X. Yao[1]; [1]Chinese Academy of Science, Institute of Cellular Dynamics, Hefei, China, [2]Physiology, Morehouse School of Medicine, Atlanta, GA

During cell division, chromosome segregation is orchestrated by the interaction of spindle microtubules with the centromere. A dramatic reorganization of interpolar microtubules into a highly organized central spindle between the separating chromatids is required for the initiation and execution of cytokinesis. Central spindle organization requires mitotic kinesins and the chromosomal passenger of Aurora B kinase complex with INCENP and survivin. The current working model argues that INCENP targets Aurora B to its points of action while the precise role of survivin is not yet known. Here we show that human survivin governs Aurora B kinase activity and its relocation to the central spindle using mitotic kinesin MKLP2. We found that survivin interacts with MKLP2 C-terminal tail and disruption of MKLP2-survivin interaction prevents the relocation of Aurora B-INCENP protein complex to the central spindle and caused cytokinesis defects. This MKLP2-mediated, survivin-dependent relocation of the Aurora B protein complex was demonstrated by a set of rescue experiments in which exogenous expression of full-length but not motor-less or tail-less MKLP2 restores the Aurora B kinase complex trafficking and faithful cytokinesis. Surprisingly, survivin-deficiency resulted in minimized

Aurora B kinase activity and aberrant targeting of mitotic kinesin MKLP1. The importance of the Aurora B phospho-regulation was validated by correct targeting of ectopic expression of a phosphorylation-mimicking mutant of MKLP1 and faithful completion of cytokinesis. These results indicate that survivin represents a link between the relocation of Aurora B kinase activity to central spindle and completion of cytokinesis.

2128

**Identification and Characterization of Genes Conferring Resistance to Signal Transduction Inhibitors in Malignant Human Mammary Epithelial Cells**

S. Moonlee, P. A. Kenny, M. J. Bissell; Cancer Biology, Lawrence Berkeley National Laboratory, Berkeley, CA

We have demonstrated previously that the malignant phenotype of a human mammary tumor cell may be reverted in 3D cultures using inhibitors of β1-integrin, EGFR and PI3K. These pathways become reciprocally crossmodulated when cells are cultured in a 3D laminin-rich extracellular matrix assay but not in 2D. In order to screen for additional pathways involved in the restoration of the breast acinar structure, we have generated a retroviral cDNA library from malignant mammary epithelial cells and screened for genes conferring resistance to the phenotypic reversion induced by inhibitors of EGFR and PI3 Kinase. Transduction of HMT3522-T4-2 cells with this library allowed us to successfully isolate several reversion-resistant colonies. We rescued the cDNA inserts from these colonies and validated the resistance phenotype by re-expression in naïve T4-2 cells. We are currently focusing on five genes. These include genes that confer resistance against EGFR inhibition or against PI3-K inhibition. We are currently determining how these proteins interact with, and modify the activities of these signaling pathways, and also determining their contribution to normal mammary acinar morphogenesis.

2129

**Human c-Src Tyrosine-phosphorylates Protein Kinase G (PKG) and PKG Serine-phosphorylates Human c-Src, Enhancing Tyrosine Autophosphorylation: Potential Mechanism of Decreased Apoptotic Cell Death in Cancer Cells**

R. R. Fiscus, C. G. Choi, J. Tsim, L. H. Leung, C. Y. Wong; Dept. Physiol. (Fac. Med.) and Mol. Cell. Gerontol., Chinese University of Hong Kong, Shatin, NT, Hong Kong Special Administrative Region of China

Our laboratory has shown that nitric oxide, natriuretic peptides and cGMP analogs, via cGMP/PKG activation, enhance cell survival by inhibiting key steps of pro-apoptotic pathway in normal mammalian cells (e.g. pancreatic islets). In transformed mammalian cells (e.g. neuroblastoma and human ovarian cancer cells) the cGMP/PKG pathway appears already activated and contributes to anti-apoptotic/pro-survival effects. Because v-Src tyrosine-phosphorylates PKG and enhances its activity, we hypothesized that, in cancer cells, higher levels of c-Src tyrosine kinase may phosphorylate PKG, increasing PKG-mediated anti-apoptotic effects. The present study, using recombinant PKG-1α and human c-Src in vitro, shows that c-Src tyrosine-phosphorylates PKG-1α (Western blot, PY20 antibody). Autophosphorylation of c-Src was enhance by PKG-1α, suggesting PKG serine-phosphorylates c-Src, enhancing Src tyrosine kinase activity. Previously, protein kinase A (PKA) was shown to phosphorylate c-Src at serine-17 and enhance its tyrosine kinase activity. Sequence at serine-17 (R-R-R-S-) suggested that c-Src could be a good substrate for PKG. To test this, we used an antibody recognizing phosphorylation at serine/threonine with arginine at -3 position (characteristic of PKA/PKG phosphorylation sites). PKG, like the positive control PKA, phosphorylated human c-Src. The data indicate human c-Src tyrosine-phosphorylates PKG-1α and PKG-1α serine-phosphorylates human c-Src, enhancing autophosphorylation. Because Src tyrosine kinase activity is elevated in many cancer cells and PKG activation inhibits onset of apoptosis, our data suggest that c-Src and PKG may work in concert, one phosphorylating the other, to inhibit apoptosis, thus promoting cell survival. Support: RGC Competitive Earmarked Grant (CUHK4169/02M), a Direct Grant and a Strategic Grant to RRF.

2130

**Lack of Natural Tumor in the Ocular Lens Is Partially Derived from the Distinct Function of the RAF/MEK/ERK Signaling Pathway**

D. W. Li, J. Liu; The Hormel Institute, University of Minnesota, Austin, MN

The ocular lens is the only organ that does not develop spontaneous tumor. The molecular mechanism for this phenomenon remains unknown. Through examination of the signaling pathways mediating stress-induced apoptosis, here we presented evidence to show that different from most other tissues in which the extracellular signal-regulated kinases (ERKs) pathway is generally implicated in mediation of survival signals activated by different factors, the RAF/MEK/ERK signaling pathway alone plays a key role in stress-activated apoptosis of lens epithelial cells. Treatment of N/N1003A cells with calcimycin, a calcium mobilizer, activates the RAF/MEK/ERK pathway through RAS, which is indispensable for the induced apoptosis because inhibition of this pathway by either pharmacological drug or dominant negative mutants greatly attenuates the induced apoptosis. Calcimycin also activates p38 kinase and JNK2, which are not involved in calcium-induced apoptosis. Downstream of ERK activation, p53 is essential. Activation of RAF/MEK/ERK pathway by calcimycin leads to distinct up-regulation of p53. Moreover, over-expression of p53 enhances calcimycin-induced apoptosis while inhibition of p53 expression attenuates calcimycin-induced apoptosis. Up-regulation of p53 directly promotes Bax expression, which changes the integrity of mitochondria, leading to release of cytochrome C, activation of caspase-3 and eventually execution of apoptosis. Thus, our results that the RAF/MEK/ERK pathway mediates stress-induced apoptosis not only provide a novel signaling pathway for calcium-induced apoptosis but also give a partial explanation for the lack of spontaneous tumor in the lens. Upon DNA damages induced by various stress factors, activation of the RAF/MEK/ERK pathway, instead of promoting survival of the cells with genetic lesions, signals apoptosis of the damaged cells. In this way, the cells bearing the genetic damages are removed in a timely manner and thus tumor development is avoided.

2131

**Isolation and Characterization of New MicroRNAs Expressed Early in Mouse Telencephalon, Exploring Their Roles in Mantainance of Neural Progenitors and Neurodevelomental Processes**

M. Zollo,[1] P. Carotenuto,[1] L. Garzia,[1] A. M. Bello,[1] N. Marino,[1] V. Aglio,[1] C. Roma,[1] V. Maffia,[1] G. Vitale,[1] J. Rubenstein,[2] A. Bulfone[3]; [1]CEINGE, Advanced Biotecnology S.C.RA.L, Naples, Italy, [2]Nina Ireland Laboratory of Developmental Neurobiology, Center for Neurobiology and Psychiatry Genetics, University of California at San Francisco, San Francisco, CA, [3]Stem Cell Research Istitute, Istituto Scientifico San Raffaele, Milan, Italy

Available data on genome sequence, expressed regions of the genome and gene expression in specific tissues is revolutionizing biological research.

A series of critical steps, ordered in a spatial and temporal time in the development of human brain, are occurring in an organized manner in order to give rise to a properly formed normal forebrain. Disorders associated to alteration on these steps are still orphan of gene identification. We aim within this functional genomic project approach to identify and characterize new genes involved in the developing mouse telencephalon and translate their functions to human so as to identify their homologues and associate them to neurodevelopmentaldisorders. We present here the full characterization of 832 transcripts preferentially expressed at E14.5 mouse telencephalon. Of this collection, 323 (39%) correspond to novel rare transcripts, which include 48 (14%) new microRNAs. These latest class corresponds to an unique collection of microRNAs precursors preferentially expressed at E.14.5 telencephalon and with a potential control function versus target genes related to cellular proliferation, migration and neural specification and differentiation. Within this collection one microRNA, 24.E3, was found highly expressed in Neural Stem Cells and thus controlling level of expression of several target genes involved in trascriptional factor activation connected to neurogenenis including cell cycle and proliferation, nuclear/cytoplam transport and a cancer associated gene. Preliminary data related to other microRNAs identified will be presented, thus representing a valuable resource for the identification of novel genes and targets involved in neurodevelopmental processes. REFERENCE: Telencephalic Embryonic Subtractive Sequences: A Unique Collection of Neurodevelopmental Genes. *Zollo et al. Journal of Neuroscience*, in press 2005. This work was supported by: Ministero Istruzione Universita´ Italiana, MIUR Ricerca-RBAU01RW82

2132

**The D806N Mutation in EphA3 Identified in Human Colorectal Cancer Causes Loss of Tumor Suppressive Functions**

E. Johnson, B. Wang; Medicine, Pharmacology, Case Western Reserve University, Cleveland, OH

Overexpression and mutation of several Eph kinases has been reported in a variety of human tumors. However, the role of Eph kinase overexpression and mutations in tumor etiology and progression remain to be elucidated. Using site directed mutagenesis and a retroviral expression system we set out to investigate the functional consequences of S792P and D806N mutations previously reported in human colorectal cancer. Wild type (WT) and mutant EphA3 were tagged with FLAG epitope in between the signal peptide and the mature sequence to enable ready detection and anti-FLAG antibody clustering of the receptors. Stimulation with either ephrinA5-Fc or M2 anti-FLAG antibody resulted in increased tyrosine phosphorylation of WT- and S792P-EphA3 in 293E cells. In contrast, similarly stimulated D806N-EphA3 failed to become phosphorylated suggesting that D806N, but not S792P mutation, caused a loss of EphA3 catalytic activity. Indeed, D806N mutant was found to be kinase-inactive in an in vitro kinase assay. These observations led us to focus on D806N mutation in colorectal cancer cells. Among colorectal cancer cell lines screened, DLD1 was chosen because of low expression of endogenous EphA kinases. Similar to our report with EphA2 kinase, ligation of WT-EphA3 on DLD1 cells decreased integrin-dependent adhesion to fibronectin and retarded wound healing. The inhibitory effects were diminished by D806N mutation. Moreover, while the overexpression of WT-EphA3 slowed DLD1 cell proliferation in three-dimensional MatriGel and planar clonal growth assays, D806N failed to suppress cell proliferation. Thus the expression and subsequent activation of EphA3 are capable of suppressing several cell behaviors associated with malignant transformation. More importantly, the acquisition of the D806N mutation abrogates the normal functions of EphA3. The loss of function mutation in human colorectal cancer may contribute to the malignant progression.

2133

**Impairment of TGFβ-Increased Stat3 Activity during Acqusition of Drug Resistance**

N. Shih,[1] S. Tseng,[1] K. Chen,[2] B. Ho,[3] Y. Lin[4]; [1]Cancer Reseach Division, National Health Research Institutes, Taipei City, Taiwan Republic of China, [2]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei City, Taiwan Republic of China, [3]Graduate Institute of Medical Science, Taipei Medical University, Taipei City, Taiwan Republic of China, [4]Department of Medicine, Fu-Jen Catholic University, Taipei City, Taiwan Republic of China

Acquired drug resistance of tumor cells has been reported to associate with insensitivity to cytotoxic cytokines. However, the molecular mechanism(s) for this cross-resistance is still obscure. We examined possible signaling alterations associated with adriamycin resistance and TGFβ insensitivity. A lung adenocarcinoma cell line, NCI-H23, resistant to adriamycin (H23ADR) is much more refractory to TGFβ-mediated growth suppression than parental cells (H23P). Upon ligand stimulation, receptor-mediated Smad2 activation and Smad-dependent transcriptional activation of a report gene could be observed in both cell lines. However, in contrast to Mv1Lu cells, the activation magnitude was not sufficient to induce growth arrest and cell death in H23P cells, indicating that TGFβ-induced growth suppression in the cells is Smad-independent event and purely attributed to increase in cell doubling time from 28.8 to 118.7 hours. Comparing activation profile of Samd2-independent signaling molecules, we first demonstrate that Stat3-mediated pathway is affected during acquisition of adriamycin resistance. TGFβ1 augmented endogenous Stat3 activity in H23P cells, but barely affected that in H23ADR cells. Consistently, MCF7 parent and its adriamycin-resistant partner also showed a similar result. Experiments using constitutively active or dominant-negative Stat3 further confirm that TGFβ-increased Stat3 activity plays a negative regulator to growth of H23 cells. Additionally, the study using H23 cells resistant to taxol, cisplatin, or 5-flurourodine demonstrates that failure response to TGFβ-augmented Stat3 activity is unique to cells acquired adriamycin resistance; whereas, intriguingly, acquired taxol resistance of H23 cell further downregulates endogenous Stat3 activity and is correlated with its cell growth. The data suggests that Stat3-mediated pathway is the major altered target during anticancer drug resistance and accounted for cells insensitive to TGFβ. Thus, the role of Stat3 in cross-resistance to adriamycin and TGFβ may provide an alternative way to monitor tumor malignancy.

2134

**SEPT6: Genomics, Transcripts, Expression Profile and Evidence of Deregulated Expression in Lymphoma**

C. B. Todd,[1] M. Dattani,[2] H. Grabsch,[2] S. S. McDade,[1] K. J. Hillan,[3] P. A. Hall,[1] H. Russell[1]; [1]Center of Cancer Research and Cell Biology, Queen's University Belfast, Belfast, United Kingdom, [2]Academic Unit of Pathology, University of Leeds, Leeds, United Kingdom, [3]Genentech Inc, South San Francisco, CA

An increasing body of data indicate that members of the septin gene family are implicated in human neoplasia. For example SEPT9 lies at a locus that shows allelic imbalance in ovarian and breast cancer, is a target for retroviral insertional mutagenesis and is over-expressed in diverse human tumours and is a translocation partner for MLL in human leukaemia. SEPT6 has also been implicated in leukaemia as a fusion partner of MLL. We therefore investigated the genomic architecture, transcript profile, mRNA and protein expression of SEPT6. The 12 exons of SEPT6 span 77.6 kbase on Xq24 and undergo complex splicing events. We defined these splicing events and report 7 distinct transcripts that encode 5 distinct

polypeptides. Of note are 3 discrete transcripts encoding the same 427 amino acid polypeptide: a phenomenon also seen with SEPT9. We used a quantitative RT-PCR strategy to define the expression of these transcripts in cell lines and in fetal and adult tissues and show tissue specific patterns of expression. We took advantage of Affymetrix probe sets from sequences in exon 11a (212415_at), 11b (212413 _at, 212414 _at and 214298_x_at) and exon 12 (213666_at) and define SEPT6 mRNA expression in normal, diseased and neoplastic human tissues (n=10360). The RT-PCR data validated the expression array and SEPT6 is expressed predominantly in lymphoid and CNS tissues. These data are further substantiated by the use of anti-SEPT6 sera we generated that show that SEPT6 expression is high in these cell types. SEPT6 protein is associated with the microtubule network and we have also identified a novel nuclear form of SEPT6 and show this to be mediated by a bipartite NLS in the C terminus of SEPT6. Finally we show that SEPT6 is consistently over-expressed in lymphoid neoplasms (n=106).

2135

**The Endolysosmal Membrane Protein Endolyn Is Up-regulated in Cancer Cells via Elevated Ap-1 Activities**

G. Zhou,[1] Y. Zhang,[2] J. Tang,[1] D. Mole,[3] S. M. Watt,[2] C. F. Campbell[1]; [1]Centre for Cancer Research and Cell Biology, Queen's Univesity of Belfast, Belast, United Kingdom, [2]Stem Cell Research Laboratory, Oxford Centre, National Blood Service, Oxford, United Kingdom, [3]Department of Surgery, Queen's Univesity of Belfast, Belast, United Kingdom

The endolysosmal membrane protein endolyn (CD164) regulates proliferation, differentiation, and migration of stem and progenitor cells but little is known about its role and transcription regulation in relation to the cancers. This study aimed to compare the expression pattern of endolyn in normal and malignant tissues and explore the underlying regulatory mechanisms. Using immunohistochemical staining, various cancerous and the corresponding normal tissue samples, collected from over a hundred patients and spotted on multiple tissue microarrays, were assessed for their endolyn expression. It was found that endolyn expression was up-regulated in almost all investigated cancer tissues (with immunostaining scores at least 3-4), compared with only basal levels in normal tissues from the same patients (scored at 0-2). Several other endolysosome related proteins, including lysosome associated membrane protein I (LAMP I), lysosome integrated membrane protein I (LIMP-II), Munc-8, and cathepsin D, were also shown to be up-regulated in cancers but generally to less extents. The up-regulation of endolyn occurs mainly at the transcription level as indicated with comparing endolyn mRNA levels in cancer and the adjacent normal tissues. A 225 bp-DNA sequence upstream the coding region of the human endolyn gene was predicted to contain a number of potential binding sites for transcription factors relevant to carcinogenesis. This region was further experimentally proved a promoter region, driving the expression of reporter sequence in several cancer cell lines. Furthermore, it was shown that the transcription complex AP-1 particularly contributes to the up-regulation of endolyn gene in cancer cells. These results suggest that endolyn deregulation, and consequently the altered endolysosomal function, may play important roles in the multiple molecular networks related to cancer development and progression.

2136

**Signaling Pathways for Troglitazone Activation of ERK1/2: Coupling to PPRE-driven Gene Expression and Ammoniagenesis in MCF-7 cells**

E. Friday,[1] R. Oliver,[2] T. Welbourne,[2] F. Turturro[1]; [1]Medicine-Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, LA, [2]Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, LA

We previously demonstrated that TRO induces a profound cellular acidosis in MCF-7 cells by inhibiting NHE-mediated acid extrusion. This inhibition was associated with marked GW9962 insensitive reduction in cellular proliferation (Clin Cancer Res 10:7022, 2004). The present study was designed to elucidate the signaling pathway(s) involved and the relationship to PPRE-driven gene expression and acidosis as reflected in cellular acidification and pH-sensitive ammoniagenesis from glutamine. Acute studies were performed on cells grown on glass chambers and intracellular pH assayed by BCECF fluorescence. Exchanger activity was monitored and expressed as $\Delta pH/\Delta t$. The levels of total- and phospho-ERK1/2 were measured by western blot analysis. Functional PPARγ activity was assessed by transient transfection with a PPARγ response element (PPRE) luciferase construct. Ammonium concentration in the media (measured by the microdiffusion method) was used as an index of chronic acidosis. Acutely, TRO (25uM) activated ERK 1/2 by 3.1 fold ($p<0.02$) within 4 minutes and was blocked by pretreatment with PD98059 and U0126. TRO also induces a severe cellular acidification ($p<0.01$). This acidification was abrogated by PD98059+U0126 pretreatment. Pretreatment with an EGFR kinase inhibitor (PD153035) prevented TRO-induced ERK 1/2 activation. This would indicate that TRO acts at either EGFR or upstream to activate ERK1/2. Chronically, TRO induced PPRE-driven luciferase expression 1.3 fold ($p<0.05$) and this effect was eliminated by PD98059+U0126 pretreatment. TRO enhanced ammoniagenesis 1.7 fold ($p<0.05$) and this effect was also eliminated by pretreatment with PD98059 + U0126. Our results are consistent with cellular acidosis and PPRE-driven gene expression largely controlled via ERK1/2 activation induced in turn by TRO acting near, or, at EGFR in MCF-7 cells.

2137

**Docosahexaenoic Acid Alters the Size and Distribution of Lipid Rafts**

R. S. Chapkin,[1] N. Wang,[2] J. R. Lupton,[3] I. A. Prior[4]; [1]Center for Environmental and Rural Health, Texas A&M University, College Station, TX, [2]Statistics, Texas A&M University, College Station, TX, [3]Department of Nutrition and Food Science, Texas A&M University, College Station, TX, [4]The Physiological Laboratory, University of Liverpool, Liverpool, United Kingdom

We recently generated nutritional data suggesting that chemoprotective dietary n-3 polyunsaturated fatty acids (n-3 PUFA) are capable of displacing acylated proteins from microdomains in vivo (FASEB J 18:1040, 2004). A primary source of very long chain n-3 PUFA in the diet is derived from fish enriched with docosahexaenoic acid (DHA, 22:6n-3). In this study, we sought to determine the effect of DHA on the size and distribution of lipid rafts in vivo. Using immunogold electron microscopy of plasma membrane sheets coupled with spatial point analysis, morphologically featureless microdomains were visualized in HeLa cells. Cells were transfected with GFP-truncated H-ras (GFP-tH is located exclusively to inner leaflet rafts) and subsequently incubated with DHA and control fatty acids, linoleic acid (LA, 18:2n-6) or oleic acid (18:1n-9) for 48 h. Univariate K-function analysis of GFP-tH (5 nm gold) revealed that the interparticle distance was significantly reduced ($p<0.05$) by DHA treatment compared to control fatty acids. These findings suggest that the plasma membrane organization of inner leaflets is fundamentally altered by DHA-enrichment. We speculate that our findings may help define a new paradigm to better understand the complexity of n-3 PUFA modulation of signaling networks. Supported in part by NIH grants CA59034, CA74552 and P30ES09106.

2138

**Functional Coupling of Endoplasmic Reticulum Signaling to the Induction of Dormancy and Drug Resistance in Human Squamous Carcinoma Cells**

A. C. Ranganathan, L. Zhang, A. Adam, J. Aguirre-Ghiso; School of Public Health, Department of Biomedical Sciences, University at Albany, Rensselaer, NY

Minimal residual disease, a stage in cancer progression where tumor cells after disseminating from the primary tumor enter into a protracted state of dormancy, has significant clinical relevance. However, the mechanisms that regulate tumor cell dormancy and its subsequent resistance to chemotherapy are understudied due to the lack of proper model systems. We have established a model of tumor dormancy in human squamous carcinoma (Hep3), where prolonged passaging of these cells *in vitro* results in a non-clonal acquisition of a dormant phenotype characterized by a spontaneous induction of p38 signaling. Here we show that constitutively activated p38 signaling pathway in dormant cells is linked to the upregulation of BiP/Grp78 expression and increased PERK phosphorylation and GADD153 promoter activation, all characteristic of an endoplasmic reticulum (ER) stress response. Further, we found that the active ER stress rather than inducing apoptosis selectively protects dormant cells from chemotherapy-induced apoptosis independent of proliferation and of P-glycoprotein mediated multidrug resistance and also favors the induction of dormancy *in vivo.* Pharmacologic or genetic inhibition of p38 activity resulted in a reversion of these changes and restored drug sensitivity and tumorigenicity. RNA interference and dominant negative expression analyses revealed that both BiP and PERK promote the survival of dormant cells and that BiP upregulation prevents Bax activation, and cytochrome-C release. Moreover, inhibition of the PERK induced eIF2α phosphorylation by the expression of GADD34, a regulatory subunit of eIF2α phosphatase PP1 resulted in the interruption of dormancy *in vivo*. We therefore propose that high p38 activation coupled to ER stress signaling confers novel survival advantage to the disseminated metastatic cells by activating the dormancy program and protecting them from stress insults such as chemotherapy.

2139

**Complete Inhibition of P-glycoprotein by Simultaneous Treatment with Modulators and the UIC2 Monoclonal Antibody**

Z. Bacsó; Biophysics and Cell Biology, University of Debrecen MHSC, Debrecen, Hungary

P-glycoprotein (Pgp) is one of the active efflux pumps that extrude a large variety of chemotherapeutic drugs from the cells, causing multidrug resistance. The conformation sensitive UIC2 mAb potentially inhibits Pgp mediated substrate transport. However in most cases this inhibition is partial, since UIC2 binds only to 10-40 % of all Pgps present in the cell membrane. The rest of the Pgp molecules assume the conformation recognized by this antibody only in the presence of certain substrates or modulators (e.g. vinblastine, cyclosporine A). Here, we have developed a simple procedure involving the combined application of modulators and UIC2 mAb, followed by removal of the modulator, achieving near 100% inhibition of pump activity by the antibody. The accumulation of various Pgp substrates, including calcein-AM, daunorubicine (DNR) and $^{99m}$Tc-hexakis-2-methoxybutylisonitrille ($^{99m}$Tc-MIBI) was completely restored by the above procedure. Remarkably, the inhibitory binding of the antibody is brought about by 10 times lower cyclosporine A (CsA) concentration than the effective concentration of CsA, when it is applied alone. We could also demonstrate this inhibition in the presence of whole mouse blood, hence *in vivo* application of this antibody appeared to be feasible. This possibility was confirmed by the dramatic increase of DNR accumulation in xenotransplanted $Pgp^+$ tumours in response to a combined treatment with UIC2 and CsA, both administered at doses barely effective when applied alone. These observations raise the possibility of a novel, specific and effective way of blocking Pgp function *in vivo*, by the combined application of modulators and a humanized form of this antibody.

2140

**Genetic Polymorphisms of the CYP1B1 Gene and Environmental Chemical Exposure in Breast Cancer Susceptibility**

J. C. Deacon, R. Goth-Goldstein, M. L. Russell; Lawrence Berkeley National Laboratory, Berkeley, CA

Polycyclic Aromatic Hydrocarbons (PAHs) are common lipophilic organic chemicals metabolized in the body through the Cytochrome P-450 1B1 (CYP1B1) enzyme pathway, where CYP1B1 metabolizes PAHs into electrophilic intermediates that can form adducts with DNA. *CYP1B1* can be strongly expressed in mammary tissue where PAH-DNA adducts can cause mutation and cancer. Single Nucleotide Polymorphisms (SNPs) are versions of genes differing by one base-pair and can differ from each other in function. Difference in catalytic function between SNPs of *CYP1B1* may extend the electrophilic presence, thus effecting cancer susceptibility. An ongoing epidemiologic study in Brazil compares *CYP1B1* genotype, *CYP1B1* expression levels, and PAH-DNA adduct levels in normal breast tissue of breast cancer patients and controls, and estimates participant PAH exposure by questionnaire. The genotyping of participants for two *CYP1B1* SNPs has produced a partial data set and is herein discussed. DNA was isolated from mammary tissues of participants and genotyped for two SNPs by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR /RFLP). PCR was used to amplify a segment of the gene and RFLP involved use of enzymes to digest SNPs for visualization by electrophoresis. 25 genotyped samples show 22 samples with SNP M1 (88%) and 8 samples with SNP M2 (33%). These M1 and M2 frequencies are higher than shown in other groups. These data represent only 25% of the study's total participants. Once all data is collected, a case v. control comparison may show correlation between these parameters and breast cancer incidence. Contributors to this study are Maira Caleffi, Ana Paula Wernz C. Muller, and Chris A. Erdmann.

2141

**Structure/Function Analysis of ErbB2's Ability to Disrupt Epithelial Apical-Basal Cell Polarity**

A. V. Lucs,[1,2] S. K. Muthuswamy[1]; [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, [2]SUNY Stony Brook, Stony Brook, NY

ErbB2/Her2/Neu is a receptor tyrosine kinase that is overexpressed in 25-30% of breast cancers and correlates with poor clinical prognosis in node-positive patients. Since ErbB2 has no known peptide ligand, we have previously developed a small molecule dimerization strategy to inducibly activate ErbB2. Using this system, we have shown that ErbB2 has the ability to transform polarized epithelial cells by promoting proliferation and disrupting the apical-basal polarity. To gain insight into the mechanism by which ErbB2 transforms polarized epithelia, we are investigating the role played by specific autophosphorylation sites in ErbB2. MDCK cells expressing mutants of ErbB2 that either lack one autophosphorylation site or possess only one autophosphorylation site are being generated and analyzed. Funding supported by DOD Breast Cancer Research Program DAMD17-03-1-0196.

2142

**LIMD1 is a Novel Regulator of Bone Homeostasis**

H. Luderer, S. Bai, E. Morgan, K. Weilbaecher, G. Longmore; Washington University in St. Louis, St. Louis, MO

Bone remodeling is regulated by the coordinated activity of two cell types, osteoclasts (OC) and osteoblasts (OB). Osteoclasts are highly migratory cells that adhere to bone causing localized bone resorption. Osteoblasts balance this destructive activity by creating new bone. Osteoblasts also contribute to OC development by secreting factors (RANKL/OPG) inducing/inhibiting OC differentiation. Therefore, these highly specialized cell types exist in a delicate equilibrium that, when disrupted, results in pathologic consequences such as osteoporosis, arthritis, and tumor metastasis to bone. Recently, the cytosolic LIM protein Ajuba was found to contribute to IL-1-induced NF-kB and AP-1 activation by influencing the assembly/activity of an atypical protein kinase C (aPKC)/ p62-sequestosome/ TRAF6 complex (Feng and Longmore, MCB, 2005). Ajuba's closest relative, LIMD1, also interacts with the aPKC/p62/TRAF6 complex, contributing to RANKL (IL-1 related)-induced OC differentiation by affecting AP-1 activation. When LIMD1-/- mice were challenged with intra-arterial tumor injections they were protected from development of bone metastasis. Similarly, in a model of serum-induced inflammatory arthritis LIMD1 -/- mice developed fewer destructive bone lesions than wild type (wt) mice. Dysfunction of OC, OB, or both could explain these results. While LIMD1-/- mice indeed exhibit an OC defect, we found, surprisingly, that they also have a defect in OB development and function. Osteoblasts isolated from LIMD1-/- mice display increased mineralization, but support reduced OC differentiation, compared to wt controls. RT-PCR analysis of gene expression profiles during OB differentiation revealed that LIMD1-/- OB secrete less RANKL and more OPG. These data suggest an important role for the Ajuba/Zyxin family of LIM proteins in maintaining bone homeostasis under stress conditions. Understanding the mechanisms involved in maintaining the equilibrium between OC and OB will undoubtedly provide novel therapeutic targets for the potential treatment of arthritis, cancer, and other diseases of the bone.

## Neuronal Diseases I (2143-2167)

2143

**CHIP Ubiquitinates Parkinson's Disease-associated L166P Mutant DJ-1 in a Chaperone-independent Manner**

J. A. Olzmann, J. W. Pridgeon, L. Li, L. S. Chin; Pharmacology, Emory University, Atlanta, GA

Mutations in the ubiquitously expressed dimeric protein DJ-1 have recently been implicated in an autosomal recessive early-onset form of Parkinson's disease (PD). Our recent work demonstrates that the PD-associated L166P mutation disrupts DJ-1 protein folding, resulting in a spontaneously unfolded protein that is catalytically inactive and is incapable of dimerization. Furthermore, we find that the L166P mutant protein is rapidly polyubiquitinated and targeted for degradation by the proteasome. The molecular mechanism by which the L166P mutant protein is specifically recognized and earmarked for ubiquitination and subsequent degradation is currently unknown. Here we report that L166P mutant, but not wild-type DJ-1, specifically interacts with the U-box type E3 ubiquitin-protein ligase CHIP (carboxyl terminus of the Hsc70-interacting protein). CHIP has been shown to cooperate with the molecular chaperone Hsp70 in the recognition and ubiquitination of several misfolded proteins. Interestingly, our data indicate that, unlike the known CHIP substrates, L166P mutant DJ-1 does not interact with Hsp70. By using an *in vitro* reconstituted ubiquitination assay with recombinant proteins, we show that CHIP facilitates the ubiquitination of L166P mutant DJ-1 in the absence of chaperones. Ubiquitinated L166P mutant proteins migrate as a high molecular weight smear, indicating that CHIP mediates the polyubiquitination of the mutant DJ-1. Our findings suggest that, in addition to the well-known chaperone-dependent E3 ligase activity, CHIP can also directly ubiquitinate some substrates in a chaperone-independent manner. Further characterization of the molecular events that control the selective ubiquitination and degradation of misfolded or damaged proteins should enhance our understanding of the pathogenesis of PD and related neurodegenerative disorders. Supported by NIH Grant AG021489 and NS047199.

2144

**Neuroprotective Function of the Mitochondrial Protease HtrA2/Omi**

K. Klupsch,[1] H. Plun-Favreau,[1] M. Martins,[2] S. Brandner,[3] J. Downward[1]; [1]Cancer Research UK, London Research Institute, London, United Kingdom, [2]MRC Toxicology Unit, Leicester, United Kingdom, [3]Institute of Neurology, London, United Kingdom

The mitochondrial serine protease HtrA2 (also known as Omi) is released from the intermembrane space during apoptosis. Once in the cytosol, HtrA2 has been implicated in enhancing cell death. However, HtrA2 knock out mice show no evidence of reduced rates of cell death. On the contrary, these animals suffer loss of a population of neurons in the striatum resulting in a parkinsonian syndrome. This phenotype suggests that it is the protease function of this protein in the mitochondria, and not its pro-apoptotic action in the cytosol, that is critical. Recently, two HtrA2 point mutations have been identified in Parkinson's disease (PD) patients. These mutations seem to result in partial loss of proteolytic activity, possibly contributing to the etiology of PD in these patients. Mammalian HtrA2 may therefore function in vivo in a manner similar to its bacterial homologues DegS and DegP, which are involved in protection against cell stress. DegS senses unfolded proteins in the bacterial periplasm, activating a proteolytic cascade that results in the transcriptional upregulation of stress response genes. Because of this homology, we decided to test the response of wild type and HtrA2 knock out cells to mitochondrial stress stimuli. Our approach involved transcriptional profiling of mouse embryonic fibroblasts (MEFs) derived from wild type and HtrA2 knock out mice. Mitochondrial stress was triggered with rotenone, a complex I inhibitor of the respiratory chain known to induce a parkinsonian phenotype in mice. Using this approach, we have identified several genes that are differentially regulated in mouse cells. We conclude that mitochondrial HtrA2 might function in a manner similar to its bacterial homologues by protecting the mitochondria under stress conditions. The compromise of this function might result in increased neuronal cell death and contribute to neurodegenerative diseases like Parkinson's disease.

2145

**Role of Parkin's Conformation in Interaction with UbcH7**

V. A. Hristova,[1] R. J. Rylett,[2] G. S. Shaw[1]; [1]Biochemistry, University of Western Ontario, London, ON, Canada, [2]Cell Biology, Robarts Research Institute, London, ON, Canada

Autosomal Recessive Juvenile Parkinson's (ARJP) is a neurodegenerative disorder that affects dopaminergic neurons of the substantia nigra. Neurodegeneration is correlated with a variety of truncation and point mutations in the 465 amino acid protein known as parkin. It has been

suggested that parkin functions as a ubiquitin ligase (E3), and in so doing can interact with ubiquitin conjugating enzymes (E2) such as UbcH7 and UbcH8. The present study focuses on the *in situ* and *in vitro* nature of parkin interactions with UbcH7 to obtain a better understanding of the role that this may have in neurodegenerative processes. Parkin expression and purification was achieved in both HEK 293 (human embryonic kidney) and SH-SY5Y (human neuroblastoma) cells, as was heterologously and endogenously expressed UbcH7. A wide range of experimental approaches were used in this study, including GST-pull down assays, immunoprecipitation (IP), and fluorescence resonance energy transfer (FRET) to monitor interaction of parkin with UbcH7. Current studies are focused on the conformational changes induced in parkin by metal ions, EDTA, and interaction with proteins such as UbcH7 by applying methods like circular dichroism. Purified parkin required for these studies has been obtained from bacterial-lysates containing the parkin-GST fusion protein. Conditions that optimize bacterial growth and parkin expression, as well as the development of a series of chromatography steps that yield pure protein, have been identified. Changes in the secondary structure of parkin's RING domains in the presence of EDTA may reveal key conformations responsible for interaction with UbcH7 and other E2 proteins.

2146

**Molecular Characterization of Pathogenic Amino Acid Substitutions within the PARK8 Gene, *LRRK2***

J. P. Taylor, J. Dachsel, S. Lincoln, I. F. Mata, J. Kachergus, J. Johnson, M. J. Farrer; Neuroscience, Mayo Foundation, Jacksonville, FL

Parkinson's disease is one of the most common neurological diseases affecting 1.8% population over 65. Genetics plays a significant role in parkinsonism as well as other forms of neurodegeneration. Pathogenic mutations in six genes are implicated in monogenic Parkinson's disease although their functional roles in the pathways of this disorder are unknown. We recently identified seven pathogenic mutations in LRRK2 (leucine rich repeat kinase 2) that maps to chromosome 12q12 (PARK8). Patients with LRRK2 mutations exhibit clinical features similar to the classic Parkinson's disease phenotype, but intriguingly their pathology is heterogeneous and may include Lewy bodies and tau inclusions, classical hallmarks of many neurodegenerative disorders. Lrrk2 is a predicted novel protein kinase. It is highly conserved among vertebrates. In addition to the MAPKKK domain Lrrk2 has predicted leucine rich repeat, Rab-like GTPase and WD40 functional domains. Here we present initial functional characterization of the Lrrk2 protein using cell biology techniques. We showed, using homology modeling to predict the structure of the Lrrk2 domains, that deleterious mutations are located within critical conserved regions of the predicted kinase and GTPase domains. We have used epitope tagged wildtype and mutant expression constructs to demonstrate that Lrrk2 subcellular localization is not altered by pathogenic mutations in BE(M17) neuroblastoma cell lines and investigated the putative kinase activity of Lrrk2 using immunoprecipitation kinase assays. We present here that Lrrk2 is capable of kinase activity but that targets in the MAPKKK pathway such as MKK 4 and 7 are not substrates for Lrrk2. Data will be presented to demonstrate the effect of pathogenic amino substitutions on Lrrk2 kinase activity. Modulation of kinase activity in LRRK2 associated Parkinson's disease represents a powerful target for future drug development; agents that promise symptomatic benefit by halting disease progression.

2147

**Investigating Potential Bacterial Sources of Dopaminergic Neuron Toxicity in *C elegans***

S. B. Memon, J. Armagost, T. Hodges, J. B. Olson, G. A. Caldwell, K. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL

Parkinson's disease (PD) involves the progressive loss of dopamine (DA) neurons from the *substantia nigra pars compacta*. Genetic forms of PD account for only 5-10% of known cases and environmental factors appear pivotal to sporadic causality. Evidence from both environmental and genetic forms of PD indicates that overloading the ubiquitin-proteasome system is a causative risk factor for PD. Furthermore, when proteasome inhibitors were injected directly into the brains of rats, PD-like symptoms resulted (McNaught et al., 2004; *Ann Neurol.* 56:149-162). Many proteasome inhibitors were originally isolated from bacterial strains within the order Actinomycetales. There is a high rate of sporadic PD among patients from rural backgrounds, where well water and farming potentially represent sources of increased exposure to Actinomycetes. To determine if exposure to Actinomycetes could cause dopaminergic neurodegeneration, we exposed the soil nematode, *C. elegans*, expressing a GFP reporter in dopaminergic neurons (*dat-1*::GFP), to four species of *Streptomyces* (*S. lividans, S. venezuelae, S. griseus*, and *S. coelicolor*). Interestingly, when *dat-1*::GFP worms were grown on *S. lividans* and *S. venezuelae*, they displayed dopminergic neurodegeneration that increased over time, wherein at 4 days exposure 52% and 59% of dopaminergic neurons were abnormal, respectively, compared to 19% of worms grown on *E. coli* OP50. Neurodegeneration was found to be specific to dopaminergic neurons following exposure to *S. lividans* and *S. venezuelae*. Currently, we are testing enriched media to determine whether neurodegeneration will be enhanced. Additionally, we are analyzing worms exposed to these *Streptomyces* strains for more generalized stress responses and are attempting to purify the factors responsible for dopamineric neurodegeneration. Future studies will include examining a combination of environmental and genetic stressors, wherein we will assay *C. elegans* with genetic backgrounds linked to PD following exposure to Streptomycetes and other Actinomycetes.

2148

**Hypothesis-based RNAi Screening in *C elegans* to Identify Genes Associated with Parkinson's Disease**

K. A. Caldwell, S. Hamamichi, M. L. Norris, G. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL

Parkinson's Disease (PD) is the second most common neurodegenerative disorder. Recent progress in understanding familial forms of PD has identified several gene products that, when mutated, may result in protein mishandling and oxidative stress. For example, mutated or overexpressed alpha-synuclein forms aggregates, and ultimately results in a formation of the characteristic protein inclusion bodies called Lewy bodies, the clinical hallmark of PD. We have exploited the advantages of the transparent nematode, *C. elegans*, to systematically screen for factors that affect alpha-synuclein misfolding in vivo. We have employed RNAi feeding methodology using bacteria producing dsRNA targeting specific genes to screen for genetic factors influencing the misfolding of a human alpha-synuclein::GFP fusion as worm age. Candidate genes implicated in the function of the ubiquitin-proteasome system (UPS), ER-associated degradation (ERAD) pathway, unfolded protein response (UPR), and autophagy were screened. Furthermore, using the mature bioinformatics available for *C. elegans*, candidate genes indentified from microarray co-expression (Stanford Topology Map) and protein interaction (interactome) data for worm homologs of PD-associated proteins, such as DJ-1, Nurr1, PINK1, UCHL-1, parkin, and LRRK2 were also tested. From over 900 total targets, we identified 17 positive genes that result in increased alpha-synuclein misfolding. As a secondary test for putative relationships to PD, we have thus far shown that 2 of these alpha-synuclein effectors can protect *C.*

*elegans* dopaminergic neurons from oxidative damage and neurodegeneration when their corresponding cDNAs are overexpressed in transgenic nematodes. Further analysis is underway to determine if these proteins also exhibit protection against alpha-synuclein dependent degeneration of dopaminergic neurons. In all, this strategy provides a paradigm for the investigation of cellular factors that influence protein misfolding and management of intracellular stress associated with human movement disorders.

2149

**Budding Yeast Model for α-Synuclein: Increased Toxicity Linked to Ubiquitin-Proteasome Pathway Dysfunction, Oxidative Stress, and E46K Mutant**

T. Vaidya, K. Brandis, N. Sharma, S. Herrera, T. Saylawala, M. Vahedi, S. Vahedi; Lake Forest College, Lake Forest, IL

Parkinson's disease (PD) is a common neurodegenerative disorder that results from the selective loss of midbrain dopaminergic neurons. Misfolding and aggregation of the protein α-synuclein, oxidative damage, and proteasomal impairment are all hypotheses for the molecular cause of this selective neurotoxicity. Here, we describe a *Saccharomyces cerevisiae* model to evaluate the misfolding, aggregation, and toxicity-inducing ability of wild-type α-synuclein and three mutants (A30P, A53T, and A30P/A53T) and we compare regulation of these properties by dysfunctional proteasomes and oxidative stress. We found prominent localization of wild-type and A53T α-synuclein near the plasma membrane, supporting known *in vitro* lipid-binding ability. In contrast, the A30P was mostly cytoplasmic, while A30P/A53T displayed both types of fluorescence. Surprisingly, these forms of α-synuclein were not toxic to several yeast strains tested. In contrast, the new familial mutant E46K was strongly toxic to several strains, and displayed both plasma membrane localization and cytoplasmic aggregates. When yeast strains mutant for the proteasomal barrel (*doa3-1*) were evaluated, delayed α-synuclein synthesis and membrane association was observed; while strains mutant for the proteasomal cap (*sen3-1*) exhibited increased accumulation and aggregation of α-synuclein. Both *sen3*-1and *doa3-1* mutants exhibited synthetic lethality with α-synuclein. When yeasts were challenged with an oxidant (hydrogen peroxide), α-synuclein was extremely lethal to cells that lacked manganese superoxide dismutase Mn-SOD (*sod2Δ*), but not to cells that lacked copper, zinc superoxide dismutase Cu,Zn-SOD (*sod1Δ*). Despite the toxicity, *sod2Δ* cells never displayed intracellular aggregates of α-synuclein. We suggest that the toxic α-synuclein species in yeast are smaller than the visible aggregates and toxicity may involve α-synuclein membrane association. Thus, yeasts have emerged effective organisms for characterizing factors and mechanisms that regulate α-synuclein toxicity. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

2150

**Fission Yeast Model for α-Synuclein: Concentration-Dependent Aggregation Without Plasma Membrane Localization or Toxicity**

K. A. Brandis, I. Holmes, S. England, L. Kukreja; Biology, Lake Forest College, Lake Forest, IL

Despite fission yeast's history of modeling salient cellular processes, it has not yet been used to model human neurodegeneration-linked protein misfolding. Since α-Synuclein misfolding and aggregation are linked to Parkinson's disease, here, we report a fission yeast (*Schizosaccharomyces pombe*) model that evaluated α-synuclein misfolding, aggregation, and toxicity and compared these properties with those recently characterized in budding yeast (*Saccharomyces cerevisiae*). Wild-type α-synuclein and three mutants (A30P, A53T, and A30P/A53T) were expressed with thiamine-repressible promoters (using vectors of increasing promoter strength pNMT81, pNMT41, pNMT1) to test directly in living cells the nucleation polymerization hypothesis for α-synuclein misfolding and aggregation. In support of the hypothesis, wild-type and A53T α-synuclein formed prominent intracellular cytoplasmic inclusions within fission yeast cells in a concentration- and time-dependent manner, whereas, A30P and A30P/A53T remained diffuse throughout the cytoplasm. A53T α-synuclein formed aggregates faster than wild-type α-synuclein and at a lower α-synuclein concentration. Unexpectedly, unlike in budding yeast, wild-type and A53T α-synuclein did not target to the plasma membrane in fission yeast, not even at low α-synuclein concentrations or as a precursor step to forming aggregates. Despite α-synuclein's extensive aggregation, it was surprisingly non-toxic to fission yeast; future genetic dissection may yield molecular insight into this protection against toxicity. We speculate that α-synuclein toxicity may be linked to its membrane binding capacity. To conclude, *S. pombe* and *S. cerevisiae* model similar yet distinct aspects of α-synuclein biology, and both organisms shed insight into α-synuclein's role in PD pathogenesis. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

2151

**Characterization of a Novel Interaction between Parkin and Ataxin-3**

T. E. Thorarinsdottir,[1] T. Fantaneanu,[1] L. Fallon,[1] G. A. Rouleau,[2] E. A. Fon[1]; [1]Centre for Neuronal Survival, Montreal Neurological Institute, Montreal, PQ, Canada, [2]Centre for Research in Neuroscience, McGill University Health Centre Research Institute, Montreal, PQ, Canada

Parkinson´s Disease (PD) is a neurodegenerative disease that involves the death of dopamine neurons and leads to devastating motor and functional impairments. One of the genes identified to cause a familial form of PD is *parkin*. *Parkin* encodes for parkin, an E3 ubiquitin ligase, and loss of parkin activity can lead to the accumulation of toxic proteins and result in the death of susceptible neurons. It is important to identify proteins that can alter parkin activity in order to develop new therapeutic targets for PD. We have identified Ataxin-3 (Atx3) as a novel parkin interacting protein, which may modulate parkin's activity. Mutations in Atx3 have long been known to result in another neurodegenerative disease, Machado-Joseph disease (MJD). MJD is the most common dominantly inherited ataxia worldwide and occasionally presents with features of parkinsonism. Using *in vitro* pulldowns and co-immunoprecipitation, we have shown that parkin and Atx3 interact in a bimodal fashion, with the N-terminal Ubiquitin like domain (UBL) of parkin interacting with Atx3 Ubiquitin interacting motifs (UIMs), as well as the C-terminal in-between-RING region of parkin interacting with the N-terminal Josephin domain of Atx3. Further, we have confirmed previous reports of Atx3 harboring deubiquitinating activity within its Josephin domain and shown that Atx3 is specifically able to deubiquitinate *in vitro* auto-ubiquitinated parkin. These results indicate a role for Atx3 in modulating parkin's E3 ligase activity and we have identified a specific substrate of Atx3 deubiquitinating activity. Further characterization of this interaction will provide important clues about the pathogenesis of the two diseases and could potentially lead to innovative new therapeutic targets.

2152

**Functional Redundancy Between Parkin and HHAri: Implications for Neurodegenerative Disorders**

S. S. Parelkar,[1] C. Kim,[1] J. Cadena,[1] Z. Wang,[1] R. Sugal,[1] B. Pazik,[2] L. M. Schwartz[1,2,3]; [1]Molecular and Cellular Biology Program, University of

Massachusetts, Amherst, MA, [2]Pioneer Valley Life Sciences Institute, Springfield, MA, [3]Department of Biology, University of Massachusetts, Amherst, MA

Some patients with Autosomal Recessive Juvenile Parkinson's disease (ARJP) carry loss of function mutations in the gene encoding the ubiquitin E3 ligase Parkin. In contrast to Parkinson's disease, surviving dopaminergic neurons in ARJP do not form Lewy bodies, abnormal cytoplasmic inclusions rich in misfolded proteins. Given the global loss of Parkin in ARJP it is not clear why phenotypic consequences are largely restricted to dopaminergic neurons. One hypothesis is that another protein with redundant Parkin functions can substitute for Parkin in most cells. Candidates for such a redundant protein should meet a number of basic criteria. First, it should be a ubiquitin E3 ligase and bind to the same E2 conjugases as Parkin. Second, it should bind to many of the same cellular substrates as Parkin. Third, it should be able to facilitate the formation of aggresomes/Lewy bodies with the same properties as Parkin-induced aggresomes. Lastly, it should be widely expressed in the nervous system but significantly reduced in dopaminergic neurons. Using biochemical, cell culture and *in vivo* assays, we have found that the Human Homolog of Drosophila Ariadne-1 (HHAri) meets all of these criteria. Also, HHARI is a component of Lewy bodies in Parkinson's disease. These data suggest that redundancy in Parkin functions may partially account for the lineage-restricted defects observed in ARJP.

2153

**Analysis of Parkin and Tau Double Mutant Mice: Parkin as a Possible Modifier of the Risk of Tauopathy**

R. Guerrero,[1] P. Navarro,[1] E. Gallego,[1] J. Avila,[2] J. G. de Yebenes,[1] M. P. Sanchez[1]; [1]Neurology, Fundacion Jimenez Diaz-Capio, Madrid, Spain, [2]CSIC/UAM, Centro de Biologia Molecular "Severo Ochoa", Madrid, Spain

*Park-2* gene mutations are frequently found in autosomal recessive-juvenile parkinsonism (AR-JP). In other familial parkinsonisms, mutations in the *tau* gene have been reported. However, heterozygous *Park-2* mutations and haplotypes and polymorphisms of both genes constitute risk factors to develop tauopathies in the normal population. Thus, a defective interaction between parkin and tau could cause neurodegenerative diseases. In mouse models, *Park-2* gene deletion or tau mutations produce mild phenotypic alterations. However, the combination of both mutations may trigger the appearance of tau pathology. To study this possibility we generated a double mutant mouse deficient for *Park-2* expression and overexpressing a human mutant *tau* transgene. We analyzed the development and behavior of these mutants, and the macro- and microscopic aspects of young brains. We also analyzed the levels of tau expression and phosphorylation in brain sections and in protein extracts of young mutant mice. Mice develop normally and do not show any clinical deficit. We did not observed evident neuronal loss throughout the brain. However, the subcellular distribution of human tau is different. Moreover, we observed accumulation of endogenous hyperphosphorylated tau in neurons of cortex and hippocampus, regions also showing reactive astrocytosis. Levels of PHF-1 phosphorylated tau are increased in protein extracts from these regions while a tendency for an increase in GFAP expression was observed in hippocampus. Thus, the combination of *Park-2* deletion with tau transgene expression in young Park$^{-}$/Tau tg mouse brains aggravates the effects produced by each isolated mutation alone. Our results suggest that parkin, apart from being involved in AR-JP, may also be a modifier of the risk of tauopathy. Moreover, it is possible that patients with sporadic tauopathies have a combination of risk factors related with parkin and tau.

2154

**Regulation of Parkinson's Disease Protein α-Synuclein Ubiquitination by Siah-1 E3 ligase**

J. T. Lee,[1] T. C. Wheeler,[2] L. Chin,[1,2] L. Li[1,2]; [1]Department of Pharmacology, Center for Neurodegenerative Diseases, Emory University School of Medicine, Atlanta, GA, [2]Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC

Parkinson's disease (PD) is a movement disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra. Dysregulation of the ubiquitin-proteasome pathway has been implicated as an important mechanism for PD pathogenesis. Lewy bodies, a pathological hallmark of PD, contain aggregated α-synuclein and ubiquitin. Point mutations (A53T, A30P, or E46K) and gene triplication of α-synuclein have been linked to early-onset autosomal dominant familial PD. Although ubiquitinated α-synuclein species have been found in Lewy bodies of PD patients, the molecular events that regulate α-synuclein ubiquitination remain poorly characterized. Here, we report that α-synuclein interacts with mammalian *seven in absentia homologue*-1 (Siah-1), a RING finger type of E3 ubiquitin-protein ligase. *In vitro* binding assays and coimmunoprecipitation studies demonstrate that α-synuclein and Siah-1 associate both *in vitro* and *in vivo.* Immunofluorescence confocal microscopic analyses reveal that α-synuclein colocalizes with Siah-1 and E2 ubiquitin-conjugating enzyme UbcH8 in mammalian cells. By using an in *vivo* ubiquitination assay, we show that Siah-1 facilitates the ubiquitination of α-synuclein, suggesting that α-synuclein is a substrate of Siah-1 E3 ligase. Interestingly, Siah-1-mediated ubiquitination does not appear to target α-synuclein for degradation by the proteasome. Further investigation of the functional consequences of Siah-1-mediated ubiquitination may provide insight into the molecular mechanisms by which ubiquitination regulates neurotransmission and neurodegeneration. Supported by NIH Grant AG021489 and NS047199.

2155

**Nardilysin Enhances α-Secretase Activity for Amyloid Precursor Protein**

Y. Hiraoka, K. Yoshida, E. Nishi; Molecular Pathology Unit, Horizontal Medical Research Organization, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Alzheimer's disease (AD) is associated with excessive deposition of amyloid-β (Aβ) peptide in the brain. The putative α-secretase cleaves the amyloid precursor protein (APP) and produces soluble fragment (sAPPα) and carboxyl-terminal fragment (C83). The cleavage occurs within the Aβ sequence, so that an enhancement of α-secretase may prevent the progression of AD. Three members of ADAMs (a disintegrin and metalloprotease), ADAM9, ADAM10 and ADAM17/ tumor necrosis factor-α converting enzyme (TACE), are the main candidates for α-secretases. Recently, we have demonstrated that nardilysin (N-arginine dibasic convertase; NRDc) enhances ectodomain shedding of heparin-binding epidermal growth factor-like growth factor (HB-EGF) through activation of TACE (manuscript in submission). In this study, we examined the effect of NRDc on α-secretase activity for APP. APP were transiently transfected into COS7 cells with NRDc or/and TACE. After 24 h of transfection, the amount of sAPPα in the conditioned medium and C83 in the cell lysates were detected by ELISA and immunoblot analysis, respectively. NRDc expression in concert with TACE dramatically increased both sAPPα and C83, compared to a sole expression of TACE. Peptide cleavage assay *in vitro* also showed that recombinant NRDc enhanced TACE-induced cleavage of the peptide substrate corresponding to α-secretase cleavage site. These results indicated that NRDc enhances α-secretase activity for APP via activating TACE. Moreover, co-expression

experiment showed that NRDc enhances α-secretase activity for APP induced by other ADAMs, such as ADAM9, ADAM10 or ADAM12. The result suggests that NRDc is an activator for wide range of ADAMs and regulates α-secretase activity. Together, our findings identify NRDc as a potent activator of α-secretases for APP, and may lead to a novel strategy for the treatment of AD.

2156

**Role of Lysosomal pH in the Degradation of Fibrillar Forms Alzheimer's Amyloid β Peptide in Microglial Cells**

A. Majumdar, N. Asamoah, D. Cruz, F. R. Maxfield; Biochemistry, Weill Medical College of Cornell University, New York, NY

Microglia are phagocytic cells that play a major role in the clearance of fibrillar forms Alzheimer's amyloid β peptide (fAβ). Microglia internalizes fAβ and delivers it to late endosomes and lysosomes, but degradation of the fAβ is slow and incomplete. To investigate the reason for this inability to degrade fAβ we monitored the degradation of fAβ by microglia and macrophages using quantitative fluorescent microscopy. We also looked into the role of lysosomal pH in controlling this fAβ degradation. **Methods:** fAβ was labeled with Cy3 dye. Microglia and Macrophage cells were pulsed with Cy3 fAβ and were chased for different time periods. After the chase periods the loss in Cy3 intensity in the cells were monitored to evaluate the extent of fAβ degradation. Microglia cells were also treated with various proinflammatory reagents and the Cy3 fAβ degradation was monitored after these treatments. For lysosomal pH measurement the cells were loaded with Fluorescein, Rhodamine double labeled dextran and pH was determined by calculating the ratio of Rhodamine to Flourescein fluorescence. **Results:** Cy3 fAβ degradation experiments show that in contrast to the microglia, macrophages can degrade fAβ efficiently and completely. Although primary culture of microglia does not degrade fAβ but when activated by proinflammatory reagents microglia start to degrade fAβ. Lysosomal pH measurements in microglia and macrophages show that microglia has alkaline lysosomes compared to the macrophages and the proinflammatory reagents which make the microglia degrade fAβ also makes the microglial lysosomes more acidic. **Conclusion:** Unlike microglia macrophages can degrade fAβ efficiently. The alkaline pH of the lysosomes of the microglia can be an important factor for this inefficiency. When activated by proinflammatory reagents lysosomes of microglia becomes acidic thus making them capable of degrading fAβ.

2157

**Tau Dependent Microtubule Depolymerization Induced by β-amyloid**

M. E. King,[1] C. G. Glabe,[2] G. S. Bloom[1]; [1]Biology, University of Virginia, Charlottesville, VA, [2]Molecular Biology and Biochemistry, University of California at Irvine, Irvine, CA

Aberrantly folded proteins that polymerize into insoluble filaments form the two histopathological hallmarks of Alzheimer's disease (AD): extracellular plaques of ß-amyloid (Aß) peptides and intracellular neurofibrillary tangles made from the microtubule associated protein, tau. A growing body of evidence suggests that AD progression involves amyloid pathology leading downstream to tau pathology, but details of this putative mechanistic connection have remained obscure. We have modeled the potential link between amyloid and tau pathologies by co-expressing YFP-tubulin and CFP-tau in CV-1 cells, exposing the cells to various forms of Aß, and monitoring cellular responses by time-lapse fluorescence microscopy. Aß was prepared in vitro to form oligomers that are recognized by a conformation-specific antibody that detects early AD histopathology in vivo. At concentrations as low as 500 nM, oligomeric and pre-oligomeric Aß42 caused microtubules to depolymerize within one hour in tau expressing cells, but had no effects on cells that did not express tau. Similarly Aß40 also caused tau-dependent microtubule disassembly, but only at 3-5 fold higher concentrations. In contrast, fibrillar Aß42 and Aß40 did not cause microtubule disassembly in tau-expressing cells, even at much higher concentrations. Microtubules were insensitive to Aß in cells expressing fluorescent forms of the tau-related proteins, MAP2c or MAP4. These collective results suggest that oligomeric and pre-oligomeric Aß, rather than fibrillar Aß deposits, are the most toxic ß-amyloid species in AD, that oligomeric and pre-oligomeric Aß compromise neuronal health by tau-dependent disruption of microtubules, and that neurons are specifically targeted in AD because they are the only brain cell type that accumulates high levels of tau.

2158

**Herpes Virus and Amyloid Precursor Protein: Co-option of Cellular Transport Machinery for Viral Egress, a Multi-disciplinary Analysis**

E. L. Bearer,[1,2] R. E. Jacobs,[3] M. P. Conley,[1,2] P. Ferland,[1] M. Jang,[1,2] I. Burgos,[1,2] J. W. Au[1,2]; [1]Pathology and Laboratory Medicine, Brown University Medical School, Providence, RI, [2]Marine Biological Laboratory, Woods Hole, MA, [3]California Institute of Technology, Pasadena, CA

Herpes simplex virus (HSV) is a neurotropic virus that must travel both into and away from the neuronal cell body to reach the mucosal epithelium where it disseminates. We have reconstituted HSV transport in both anterograde and retrograde directions in the giant axon of the squid. We found that the virus interacts with cellular amyloid precursor protein (APP) with high affinity, with an average of 3,000 copies of APP associated with isolated viral particles displaying anterograde transport capability. By conjugating the cytoplasmic domain of APP to 100- nm fluorescent beads we have identified a "zipcode" for the synapse, a 15 amino acid peptide that is sufficient to interact with the axoplasmic transport system and deliver beads to the presynaptic terminal. APP-conjugated beads interact with high affinity to kinesin heavy chain in rat brain extracts ($K_D$ ~ 5 nM). The sequence of the APP peptide is highly conserved throughout phylogeny, with 13/15 identities between squid and human. Using deconvolution microscopy for quantitative intensity analysis of fluorescence images, we now show that HSV infection up-regulates APP expression. Nascent viral particles co-localize with APP in infected cells, with initial associations in the Golgi region but others apparently occurring throughout the egress pathway. We are now exploring micro-MRI to image viral trajectories in the living brains of mouse models through development of novel MR contrast agents and high resolution detection schemes. These results suggest that APP is a cargo motor receptor for HSV egress, and that HSV infection may be a risk factor for Alzheimer's disease. Supported by NINDS, NIGMS, The Moore Foundation, and the Dart Foundation.

2159

**Alzheimer's Dementia is Due to Faulty, Endoplasmic Reticulum (ER), Posttranslational, Protein Processing**

J. L. Holtzman,[1] R. R. Erickson,[1] L. M. Dunning,[1] D. A. Olson,[2] S. J. Cohen,[3] A. T. Davis,[4] W. G. Wood,[1] R. A. Kratrzke[5]; [1]Pharmacology, University of Minnesota, Minneapolis, MN, [2]Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, [3]Anesthesia, University of Minnesota, Minneapolis, MN, [4]Research, Veterans Administration Medical Center, Minneapolis, MN, [5]Medicine, University of Minnesota, Minneapolis, MN

**Background:** The β-amyloids (abetas) (MW ≥ 4.7 kDa) are the major components of the plaque observed in the brains of Alzheimer's patients.

The conundrum is that although they are produced in everyone during normal, ER, posttranslational processing of the amyloid precursor protein (APP), deposits are only seen in the brains of the elderly. Since in plaque the abetas are present as the free peptides, this observation would suggest that normally they are bound to carrier proteins. **Methods:** We analyzed by immunoblot with multiple antibodies the abetas in cerebrospinal fluid from normal humans. **Results:** In SDS-reducing gels all the detectable abetas have a Mr = 62 kDa and are bound to ERp57. The complex was stable when treated with neat formic or TFA acids. On the other hand, when these acids have been used to solubilize plaque, only the naked abetas have been reported. The complex was hydrolyzed by base (pH 9.0). In native gels the abetas chaperone complex had a Mr = 130 kDa and contained both ERp57 and calreticulin. **Conclusions:** Our data indicate that abetas deposits result from faulty, ER, posttranslational processing of APP with the failure to form a complex with the chaperones. This may be due to decreased levels of the chaperones. Alternatively, since ERp57/calreticulin only bind N-glycosylated proteins, there may also be decreased N-glycosylation with aging, as has been reported from other laboratories. Since the initiation, consolidation and retrieval of memory are dependent upon the continued synthesis of synaptic, membrane proteins, if their processing is similarly affected, it could explain the failure to form new synapses leading to the dementia characteristic of Alzheimer's disease.

2160

**Neuroendocrine Innate Immunity as an Early Marker of Alzheimer's Disease: Results from a 18-Month Follow-up Study**

P. Prolo,[1] F. Chiappelli,[1] A. Angeli,[2] P. Perotti,[2] M. Pautasso,[3] M. Sartori,[2] A. Dovio,[2] T. Fraccalini,[4] L. Saba,[2] S. Mussino,[2] F. Fantò,[4] M. Rosso,[5] C. Mocellini,[5] E. Grasso[5]; [1]Dentistry, UCLA, Los Angeles, CA, [2]Internal Medicine, University of Turin, A.S.O. S. Luigi Gonzaga, Orbassano, Italy, [3]Flow Cytometry, University of Turin, A.S.O. S. Luigi Gonzaga, Orbassano, Italy, [4]Geriatrics, University of Turin, A.S.O. S. Luigi Gonzaga, Orbassano, Italy, [5]Neurology, A.S.O. S. Croce & Carle, Cuneo, Italy

We designed a longitudinal study to address NK cell activity as a prognostic factor in Alzheimer's disease (AD). We followed 18 subjects (61-84 years old) with mild AD under treatment with Donezepil (10 mg/daily), and 10 healthy control subjects (59-84 years old). Change in time for MiniMental State Examination (MMSE) was analyzed in parallel to immunophenotypic parameters of T cells, and response of NK activity to physiological modulation. Psychoimmune measures were obtained at entry and at six months intervals for 18 months. NK activity was assessed untreated and after modulation by cortisol at $10^{-6}$M. We tested augmentation of NK cytotoxicity by interleukin (IL)-2 (100 IU/ml). Neither the percent of NK cells nor baseline NK cell activity were different in AD compared to controls at entry. Response to modulation by cortisol was significantly greater in AD. Change in circulating CD3+Dr+ population with time in AD was inversely correlated with the change in time in the response of NK cytotoxic activity to cortisol. Based on change in MMSE score at entry and at 18 months, AD subjects were assigned to a "fast progression" ($\Delta > 2$ points) or to a "slow progression" group ($\Delta \leq 2$ points). Change in response of NK cytotoxic activity to cortisol, and the association of this parameter with circulating activated T cells in time was greater in Fast Progression, compared to Slow Progression AD. Our findings reveal an increased sensitivity to cortisol in AD, which can be detected at the level of the cellular compartment of the immune system. Results suggest that changes in the response of NK cells to negative (e.g., cortisol) or positive modifiers (e.g., IL-2) follow progression of AD, and might be an adequate early marker of AD.

2161

***In Vitro* Activity of the γ-secretase Complex is Dependent on the Nature of Lipid Environment**

S. Zhou, H. Zhou, P. J. Walian, B. K. Jap; Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

γ-secretase was first recognized through its role in the production of Aβ peptides that are pathogenic in Alzheimer's disease (AD). γ-Secretase is a membrane protein complex with unusual aspartyl protease activity that cleaves a variety of type I transmembrane proteins, such as APP, CD44, DCC, ErbB4, E-cadherin, LRP, N-cadherin, Nectin-1, and Notch, within their transmembranous regions; therefore, in addition to its role in AD, γ-secretase has been found to participate in many other important biological functions, such as cell fate decision, cell-cell connections and intracellular signaling. Purification and characterization of active γ-secretase complex, and the availability of in vitro activity assays are crucial steps toward elucidating its molecular mechanism. We have initiated investigations into how different factors, such as detergent removal and lipids used, modulate the in vitro activity of γ-secretase complex. In our experiment the in vitro activity of γ-secretase is evaluated based on the cleavage of a synthetic fluorogenic peptide substrate containing a C-terminal fragment of APP. To complement the activity assay, we have also been examining the formation of proteoliposomes under different reconstitution conditions by electron microscopy. We have found that in vitro activity of γ-secretase complex directly correlates with the degree of proteoliposome reconstitution. Taken together, these observations indicate that the γ-secretase complex requires an appropriate lipid bilayer environment for its activity in vitro. This work was supported by the National Institutes of Health.

2162

**Biochemical Characterization of Ubiquilin: Dimerization and Interaction with Presenilin-2**

D. L. Ford, M. J. Monteiro; Biochemistry, University of Maryland, Baltimore, Baltimore, MD

Mutations in presenilin (PS1 and PS2) genes are associated with early-onset Alzheimer's disease. The presenilin proteins have been implicated in various cellular processes, including early embryo development, proteolytic cleavage of amyloid precursor protein, protein trafficking, cell cycle control, neuronal differentiation and apoptosis. Previously we reported that presenilins interact with ubiquilin, a highly conserved protein containing ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains. In addition to presenilins, ubiquilin interacts with several other proteins, and this interaction appears to regulate stability and degradation of its binding partners. We have investigated the mechanism of interaction of ubiquilin with presenilin-2. Overexpression of ubiquilin-1 leads to an increase in PS protein levels. Since ubiquitin (UB) binds substrates through isopeptide lysine linkages, and UBA domains of proteins are known to bind ubiquitin chains, we investigated whether mutation of lysine residues in PS2 would alter interaction with ubiquilin. A yeast 2-hybrid system and beta-galactosidase assay were utilized, using PS2-loop and -COOH terminal regions as bait and ubiquilin as prey. Results indicate that ubiquilin interaction with PS2 is not fully dependent on ubiquitination of PS2 polypeptides. Interestingly, we found that mutation of residues within the carboxy-terminus of PS2 affects interaction with ubiquilin. Some of these residues are predicted to be critical for presenilin function and stability. We also demonstrate that ubiquilin is capable of self-interaction, which may serve a regulatory role in ubiquilin's interaction with presenilins and other proteins. This interaction does not appear to require ubiquilin's UBA and UBL domains; rather it is dependent upon the central region of ubiquilin. We further show that ubiquilin forms dimers, and have

obtained preliminary evidence suggesting the monomer is likely to be the active form that is involved in binding other proteins.

2163

**Effect of Cortistatin on Tau Phosphorylation**

A. Rubio,[1] M. Perez,[1] L. Lecea,[2] J. Avila[1]; [1]Centro de Biologia Molecular Severo Ochoa, Madrid, Spain, [2]Molecular Biology, The Scripps Research Institute, La Jolla, CA

The principal cytoskeletal components in a neuron are the microtubules, formed by tubulin and microtubule-associated proteins (MAPs). These MAPs stabilize microtubules and may determine the complex morphology of a neuron. Tau is a MAP capable of forming aberrant fibrillar polymers when it is hyperphosphorylated in pathological situations such as Alzheimer´s disease (AD). AD is a senile dementia characterised by short memory acquisition and consolidation deficits. These deficits have been correlated with the development of the disease: first, entorhinal cortex and hippocampus (where probably occurs memory acquisition) are affected and, afterwards, a progressive degeneration is found in cortical neurons (where probably occurs memory consolidation). Moreover, the recently described neuropeptide cortistatin, whose expression is restricted to gamma amino butyric acid (GABA)-containing cells in the cerebral cortex and hippocampus, may be involved in the modulation of memory, probably by regulating the transition of information (through the sleep) from the hippocampus to the cortex. The purpose of this work is to indicate the effect of cortistatin on tau phosphorylation at specific sites, recognized by 12E8 and tau1 antibodies. It has been proposed that phosphorylation at these two sites could affect the interaction of tau with microtubules. Treatments with cortistatin of primary cortical cultures, from mice, reveal a slight decrease in tau1 immunoreactivity and a progressive increase in tau phosphorylation at the site recognized by 12E8. We also characterized tau phosphorylation in a mouse that do not express the cortistatin peptide (CST -/-). Our data suggest that tau1 immunoreactivity is higher in CST -/- mice than in control mice. In contrast, tau phosphorylation at the site recognized by 12E8 is lower in CST -/- mice. In conclusion, cortistatin seems to increase tau phosphorylation at 12E8 and tau1 sites suggesting that tau release from microtubules is facilitated.

2164

**Induction of Abnormal Phosphorylation of Tau Requires Sequential or Simultaneous Action of GSK3beta and CDK5**

M. Hashiguchi; Department of Physiology, Tokyo Medical University, Tokyo, Japan

One of the pathological hallmarks of Alzheimer's disease (AD) patient is abnormally phosphorylated tau protein. AT100, an AD-specific tau antibody, recognizes a particular set of phosphorylated residues of the pathological tau. These phosphorylation cannot be achieved by a single tau kinase, but multiple tau kinases are obviously required. Two powerful tau kinases, GSK3beta and CDK5, are found to co-exist in tau phosphorylation protein complex in brain. It seems that the two kinases phosphorylate tau simultaneously. We conducted in-vitro kinase assay using recombinant tau, p35-CDK5 and GSK3beta. Phosphorylation status of tau was analyzed by two-dimensional phosphopeptide mapping. Two peptides, Ser202/T205 and Ser396/Ser404, were phosphorylated by either GSK3beta or CDK5, while other peptides, as revealed by spots on the 2D-map, were independently phosphorylated. Simultaneous application of p35-CDK5 and GSK3beta gave seven major spots. The same spots were shown by the sequential application of p35-CDK5 and then GSK3beta. However, sequential application in the reverse order gave only six spots, and one is missing. These results suggest that the tau in the brain phosphorylation complex is sequentially processed by closely located two kinases.

2165

**Evidence for Amyloid Induced Transport Defects Initiated by Mechanical Blockage within Neuronal Processes**

S. B. Shah,[1] L. S. B. Goldstein[1,2]; [1]Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA, [2]Howard Hughes Medical Institute at UCSD, La Jolla, CA

Dystrophic neurites surrounding amyloid plaques are a hallmark of late-stage Alzheimer’s. At earlier stages of disease progression, animal models display organelle-filled swellings along their neurites, suggesting constriction of typical neuronal motor-driven transport pathways. These models also reveal increases in the degradation-resistant beta-amyloid (Aβ) 1-42, observed in several stages of fibrillization near plaques and swellings. We tested the hypothesis that Aβ is responsible for the development and/or exacerbation of these transport defects, and that transport defects result from an early event of mechanical blockage. We induced swellings in mouse neuroblastoma and hippocampal neurites via internalization of artificial obstructions (fluorescent beads of varying diameter: 20nm-500nm) or Aβ in varying fibrillization states (i.e., with different geometries). Percentages of neurites exhibiting swellings were quantified at various time points (1-16 hours) after cell exposure to the impediments. Electron microscopy confirmed bead internalization. Bead diameter and the percentage of neurites exhibiting swelling showed a positive correlation, particularly at later time points. Neurites exposed to fibrillar Aβ displayed a similar swelling phenotype in a dose- and time-dependent manner, while peptide Aβ induced cell necrosis. Quenching of FITC-Aβ fluorescence confirmed Aβ internalization. Real-time imaging of neurites exposed to beads >200nm allowed insight into swelling formation dynamics. Within one hour of exposure to non-fluorescing beads, several neurites displayed swellings consistent with our observations in fixed cells. Partial neurite retraction was followed by dramatic shifts in the cytoskeleton, resulting in decreased neurite diameter adjacent to the swellings, and sometimes, motion of swellings themselves. Probability of swelling induction was enhanced by reactive oxygen species generated following fluorescing bead excitation, but was not enhanced in the presence of the oxygen depleting agent Oxyrase. Kinematic analysis of organelles tagged with APP-YFP confirmed transport impairment as swellings developed. Support: NIH, HHMI.

2166

**Metalloendoprotease Cleavage Triggers the Final Stage of Gelsolin Amyloidogenesis**

L. J. Page, J. Suk, M. E. Huff, J. W. Kelly, W. E. Balch; Department of Cell Biology-MB6, The Scripps Research Institute, La Jolla, CA

Amyloid diseases like Alzheimer’s and Familial amyloidosis of Finnish type (FAF) stem from endoproteolytic cleavage of a precursor protein to generate amyloidogenic peptides that accumulate as amyloid deposits in a tissue specific manner. FAF patients deposit both 8 and 5 kDa peptides derived from mutant (D187Y/N) plasma gelsolin in the extracellular matrix (ECM). The first of two aberrant sequential proteolytic events is executed by furin to yield a 68 kDa (C68) secreted fragment. We now identify the metalloprotease MT1-MMP, an integral membrane protein active in the ECM, as a protease that processes C68 to the amyloidogenic peptides. We propose a pathway in which aberrant cleavage in the

secretory pathway followed by proteolytic processing within the distinct chemical environment of the ECM regulates the locale and extent of amyloid deposition and defines the distinctive disease pathology of FAF.

2167

### In Situ Small Particulate Assemblies of AA Protein in Experimental Murine AA Amyloid

S. Inoue; Anatomy and Cell Biology, McGill University, Montreal, PQ, Canada

Concerning the toxicity of amyloid proteins, soluble small oligomers of beta amyloid protein were reported to be toxic. In this study, experimental murine AA amyloid tissue was examined ultrastructurally and with immunolabeling in an effort to identify in situ small multimers of AA protein. Pieces of mouse spleen with experimental AA amyloid were fixed in a solution containing 96% ethanol, 1% glacial acetic acid and 3% water, and thin sections were stained with uranyl-lead. AA protein was partially present in a previously unreported form, that is, 6-9 nm wide particulate structures. Each particle was made up of the close parallel association of approximately 6 “helical rods” (tight helices of 1 nm wide filaments) each 5 nm long and 2-3 nm wide. Slight loosening of helices caused variations in the particle size, but when all helices in a particle were thinnest (2 nm wide), particles were flat hexagonal prisms 5 nm high and 7 nm wide made up of the close parallel packing of 6 helical rods with a tiny central pore filled with dark stain. The particles were scattered in the extracellular space, and were also closely associated with the membrane of degrading reticular cells. Their role in amyloid formation is not clear. Amyloid proteins are known to be capable of forming ion-permeable channels in lipid bilayer membranes, and murine SAA2.2 was shown to form 8 nm wide hexagons (hexamers) with a putative central pore. There is a certain similarity between particles in this study and these hexamers. A possibility is that these newly found AA protein particles are related to the formation of toxic membrane channels.

## Other Diseases II (2168-2186)

2168

### SNARE Protein Regulates *Salmonella* Intracellular Survival

S. Dai, D. Zhou; Biological Sciences, Purdue University, West Lafayette, IN

After entering host cells, *Salmonella enterica* Typhimurium resides within membrane-bound vacuoles where it survives and replicates. *Salmonella*-containing vacuoles (SCVs) have been shown to undergo a series of maturation steps including sequentially acquisition of early and late endocytic pathway markers. *Salmonella* is able to avoid direct fusion with lysosome. To understand the underline mechanism of how *Salmonella* achieve this, we examined roles of syntaxin 7, syntaxin 8, and VAMP7 in *Salmonella* invasion and intracellular replication. Immunofluorescent staining showed increased localization of syntaxin 7 and VAMP7 on SCVs and Sifs from 6h to 24h P.I., while syntaxin 8 only localized on early SCV (2h P.I.) in both HeLa and RAW264.7 cells. The exogenously expressed wild-type or dominant negative form of Syntaxin 7 and syntaxin 8 had no significant effect on *Salmonella* replication in HeLa cells. In contrast, wild-type VAMP7 decreased *Salmonella* intracellular replication and dominant negative VAMP7 increased it. However, wild type or dominant negative VAMP7 had no obvious effect on the intracellular survival of SPI2 mutant in HeLa cells. Our study suggests that VAMP7 is involved in intracellular bacterial survival and might be a target of *Salmonella* SPI2 effector proteins.

2169

### A Single Domain Mediates both SPI-1 Type III Secretion and Translocation of *Salmonella* SopA

W. M. Higashide, D. Zhou; Biological Sciences, Purdue University, West Lafayette, IN

*Salmonella enterica* serovar Typhimurium encodes two type three secretion systems (TTSSs) within the *Salmonella* pathogenicity island 1 (SPI-1) and island 2 (SPI-2). These type III protein secretion and translocation systems function to inject a panel of bacterial effector proteins into host cells to promote bacterial entry and subsequent survival inside the host cells. Effector proteins contain secretion and translocation signals that are often located at their N-termini. We have determined that the N-terminal 45 amino acid residues of *Salmonella* SopA are necessary and sufficient for directing its secretion and translocation through the SPI-1 TTSS. Interestingly, $SopA_{1-45}$, but not $SopA_{1-44}$, is also able to bind to its chaperone InvB, implying that the SPI-1 type III secretion and translocation of SopA requires its chaperone. Our data demonstrates that the secretion and translocation signals of *Salmonella* SopA can not be separated and may even be the same domain. We speculate that the SPI-1 type III secretion and translocation of SopA may be coupled.

2170

### *S pombe* as a Model System for the Investigation of *Yersinia* Outer Membrane Proteins (Yops)

D. J. Wiley, K. Schesser; Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL

The pathogenic yersiniae (*Y. pseudotuberculosis*, *Y. pestis* and *Y. enterocolitica*) utilize a type three secretion system to inject effector proteins, known as *Yersinia* outer membrane proteins (Yops), into host cells to evade host immune responses. These Yops are believed to contribute to yersiniae’s virulence by directly modulating host factors. However, the identification of specific host factors and the biological significance of their modulation have been hampered by the lack of a good discovery model system. Here, we identify fission yeast, *Schizosaccharomyces pombe*, as an ideal model system to investigate the functions and molecular targets of the Yops. Fission yeast have a highly defined pattern of growth and a cytoskeleton that is easily visualized and quantified by microscopy, which is especially relevant for Yops since most have been shown to affect the cytoskeleton. We have used *S. pombe* to study the function of the kinase domain of *Yersinia* protein kinase A (YpkA). In mice the kinase domain of YpkA is important for virulence, however, in model systems with YpkA being expressed at high levels the kinase domain appears to be dispensable. Our results show that the kinase domain of YpkA contributes to the disruption of the actin cytoskeleton in *S. pombe* and demonstrates the importance of expression level and duration when analyzing virulence activities. Moreover, we believe that *S. pombe* is well-suited for biochemical and genetic studies, including mutagenesis and suppression screens, which lend themselves to the application of identifying the pathways for each Yop’s role in *Yersinia’s* virulence.

2171

### Lipid Membrane Rafts Mediate *Pseudomonas aeruginosa* Internalization in Corneal Epithelium Following CTL Wear *in vivo* and *in vitro*

H. D. Cavanagh, N. Yamamoto, D. M. Robertson, W. M. Petroll; Ophthalmology, UT Southwestern Medical Center, Dallas, TX

**PURPOSE:** Corneal epithelium (CE) responds inversely to hypoxia induced by contact lens wear (CTLW) with increased binding and internalization of *Pseudomonas aeruginosa* (PA). The purpose of this study was to determine if membrane lipid rafts (MLR) play a role in this process *in vivo, in vitro* and following CTLW. **METHODS:** MLR formation was evaluated *in vivo* in rabbit CE with or without CTLW; immortalized human CE cells (hTERT) were used *in vitro*. MLR were detected by FITC-labeled β-subunit cholera toxin known to bind MLR component GM1 with multiphoton confocal microscopy. Three pathogenic PA strains were tested; internalization was assessed by gentamicin survival assay and confirmed by pretreatment of h-TERT cells with three cholesterol metabolism inhibitors. Cytotoxic effects of inhibitors were excluded by live-dead assays. Specific interaction of PA and MLR was confirmed by FACS analysis. **RESULTS:** Normal rabbit CE does not exhibit MLR *in vivo*; these are induced by PMMA CTLW, but not by PA exposure alone to all three test strains. PA exposure after PMMA CTLW showed binding to MLR-forming cells *in vivo* followed by MLR aggregation and internalization for all strains. A similar sequence of MLR formation and PA internalization was seen in hTERT cells. Internalization of all strains was blocked in a concentration dependent manner by cholesterol metabolism inhibitors ($P < 0.01$); cytotoxic effects were excluded by live-dead assays over concentrations studied. FACS analysis showed exposure of two PA test strains to cell lysate significantly increased fluorescence intensity, demonstrating binding of MLR-GM1 to PA. **CONCLUSIONS:** These findings demonstrate for the first time that CTLW-mediated PA internalization involves MLR platforms. These findings offer a unique new strategy for prevention of CTL-related PA infection by blocking MLR formation.

2172

**Differential Requirement for Syk and PI 3-Kinase in the Opsonin-Independent Engulfment of Pathogenic *Neisseria* by Neutrophil-Restricted CEACAM3**

H. Sarantis, S. E. McCaw, S. D. Gray-Owen; Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON, Canada

While pathogenic *Neisseria* species (*Neisseria gonorrhoeae*, *Neisseria meningitidis)* attach to epithelia and other human tissues via carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs), neisserial binding by the neutrophil-restricted CEACAM3 allows potent opsonin-independent bacterial engulfment and subsequent killing. Upon CEACAM3 engagement by gonococci, the receptor's cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) is phosphorylated by Src-family kinases, allowing phosphatidylinositol 3-kinase (PI3K) activation, F-actin assembly, and bacterial engulfment. In this study we sought to determine if the tyrosine kinase Syk was involved in the initiation of these signaling events. Because Syk is restricted to hematopoietic cells, we used a recombinant Syk-EGFP chimera to pursue studies in a transfected HeLa cell model. We have observed that Syk-EGFP associates with CEACAM3 and is phosphorylated during gonococcal infection of HeLa-CEACAM3 as well as human neutrophils. Furthermore, using genetic mutants of CEACAM3, we have shown that CEACAM3-Syk association is ITAM-dependent. Contrary to FcR (Fc receptor)-mediated phagocytosis, Syk-EGFP did not increase gonococcal uptake by HeLa-CEACAM3, and the Syk inhibitor piceatannol did not alter gonococcal uptake in human neutrophils. We have, however, observed that HeLa-CEACAM3 internalization of larger particles (5.6 μm anti-CEACAM IgG-coated beads) absolutely requires both Syk-EGFP and PI3K. This suggests the role of Syk in CEACAM3-mediated uptake involves membrane trafficking to sites of phagocytosis to allow sufficient pseudopod extension for phagosomal closure. Because gonococci are relatively small in size (~1 μm), encolusure of the bacteria-containing phagosome can occur in the absence of Syk, whereas engulfment of bacterial aggregates requires significant pseudopod extension. The close parallels that continue to be unravelled for CEACAM3- and Fc receptor-mediated engulfment depict a highly specialized, opsonin-independent means to capture and destroy CEACAM-binding pathogens, suggesting a highly evolved paradigm of specific innate immunity.

2173

**Analysis of the Penicillium marneffei Secretome: the Proteomics of an Opportunistic Pathogen**

G. R. Walker,[1] S. Pizzino,[1] K. Segna,[2] T. Kim,[1] C. Cooper[1]; [1]Biological Sciences, Youngstown State University, Youngstown, OH, [2]Biological Sciences, Christopher Newport University, Newport News, VA

Penicillium marneffei is an opportunistic fungal pathogen that exhibits a pronounced dimorphism. This fungus can exist as either a multicellular mould form or a single celled yeast form. The latter entity is the cause of penicilliosis in immune-compromised individuals. Opportunistic organisms secrete a variety of proteins (secretome) that are necessary for maintaining their structure as well as modifying their extracellular environment enabling them to survive in the host. Our laboratories have been applying proteomics to the problem of fungal dimorphism . Electrophoretic analysis of the P. marneffei secretome shows differential expression of proteins between the two growth forms. Past studies have shown that catalase activity, potential component of the secretory repertiore, is much higher in the yeast form, possibly enabling the organism to combat the superoxide attack of macrophages and may be part of the secretory repretoire of the cell. This study shows that there is a major yeast specific secretory protein with a molecular weight similar to that for catalase. Based on its mobility in two-dimensional electrophoretic gels, the protein has a molecular weight of approximately 65 kDal and a pI of approxiamately 4.0-4.5. Pre-fractionation by liquid isoelectric focusing supports the basic characteristics of this major yeast secretory protein. Further analysis of this is being carried out using mass spectroscopy. The identification of phase specific protein in the secretome will give us a better understanding of the mechanism of fungal dimorphism as it relates to pathogenesis.

2174

**Internalization of Group A Streptococcus into Primary Oropharyngeal Keratinocytes Involves Clathrin and Cholesterol**

A. P. Hakansson,[1,2] M. R. Wessels[1,2]; [1]Division of Infectious Diseases, Children's Hospital Boston, Boston, MA, [2]Harvard Medical School, Boston, MA

Group A streptococci (GAS) can be internalized by epithelial cells in vitro. We have previously shown that expression of streptolysin O (SLO) helps the bacteria avoid ingestion by epithelial cells. SLO-negative GAS are internalized in 50-fold higher numbers than SLO-producing bacteria, are quickly seen inside vesicles stained for lysosomal markers including synaptotagmin VII (SytVII), and are rapidly killed through a pH-dependent mechanism. Internalized SLO-positive GAS avoid trafficking to lysosomes and are killed in a slower fashion by a mechanism independent of pH. These results prompted a more complete investigation of the mechanism(s) used for internalization of GAS and how SLO modulate this process. A possible direct internalization of SLO-negative bacteria into lysosomes made us investigate the involvement of clathrin as synaptotagmins have been shown to contain clathrin-dependent internalization motifs. We also investigated the role of cholesterol as SLO is a

cholesterol-dependent toxin that may modulate internalization by interacting with cholesterol-rich membrane domains. Inhibition of clathrin-mediated uptake by monodansylcadaverin or chlorpromazine effectively reduced the internalization of both SLO-positive and negative GAS by 80-90%. In contrast, depleting cholesterol from the membrane with methyl-β-cyclodextrin or adding the sterol-binding molecule nystatin only inhibited the internalization of SLO-negative GAS (90%) without affecting internalization of SLO-positive GAS. These results suggested that SLO interferes with cholesterol-dependent internalization in a fashion similar to drugs that deplete or bind cholesterol. This hypothesis was supported by the finding that addition of SLO to cell monolayers caused a 70% reduction of internalization of SLO-negative GAS. Together, the findings suggest that GAS can be taken up through two different pathways, both of which require functional clathrin. One of these pathways also depends on the presence of cholesterol in the membrane and is inhibited by SLO.

2175

**Degenerative Effect of PepA from *Helicobacter pylori* on Gastric Epithelium Cell**

S. Yun,[1] S. Lee,[2] C. Uhm,[2] S. Hwang[1]; [1]Department of Bioscience, School of Life Science and Biotechnnology, Korea University, Seoul, Republic of Korea, [2]Department of Anatomy, College of Medicine, Korea University, Seoul, Republic of Korea

Aminopeptidase a/i (PepA) from *Helicobacter pylori* is first purified, cloned and expressed in *E.coli.* The cloned enzyme has the same enzymatic properties as the wild-type enzyme. PepA is 54.965kDa, homohexamer and it has amidolytic activity against PSP(L-phenylalanyl-3-thia-phenylalanine); the release of thiophenol upon enzymatic hydrolysis of PSP(L-phenylalanyl-3-thia-phenylalanine) is spectrophotometrically detected with the aid of 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB). The enzyme is activated by $Mg^{2+}$, $Mn^{2+}$ ,or $Ni^{2+}$ and inhibited by ion chelators. AGS gastric epithelial cells are treated with PepA to investigate cellular changes by electron microscopy. We observed ultrastructural and morphological changes on gastric epithelium cells. The results suggest the potential use of PepA as a drug target to develop novel anti-H. pylori agents.

2176

**Recognition and Ubiquitination of *Salmonella* Type III Effector SopA by an Ubiquitin E3 Ligase, HsRMA1**

Y. Zhang, W. Higashide, S. Dai, D. Sherman, D. Zhou; Biological Sciences, Purdue University, West Lafayette, IN

*Salmonella* translocate bacterial effectors into host cells to confer bacterial entry and survival. It is not known how the host cells cope with the influx of these effectors. We report here that the *Salmonella* effector, SopA, interacts with host HsRMA1, an ubiquitin E3 ligase with a previously unknown function. SopA is ubiquitinated and degraded by the HsRMA1-mediated ubiquitination pathway. A *sopA* mutant escapes out of the *Salmonella*-containing vacuoles less frequently to the cytosol than wild type *Salmonella* in HeLa cells in a HsRMA1-dependent manner. Our data suggest that efficient bacterial escape into the cytosol of epithelial cells requires HsRMA1-mediated SopA ubiquitination and contributes to *Salmonella*-induced enteropathogenicity.

2177

**Apoptosis Triggered by Rv1818c, a PE Family Gene from *Mycobacterium tuberculosis* is Regulated by Mitochondrial Intermediates in T cells**

B. N. Kithiganahalli; Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India

Ectopic expression of the *Mycobacterium tuberculosis* PE-family gene *Rv1818c,* triggers apoptosis in the mammalian Jurkat T cell line, which is blocked by the anti-apoptotic protein Bcl-2. Although complete overlap is not observed, a considerable proportion of cellular pools of ectopically expressed Rv1818c localizes to mitochondria. However, recombinant Rv1818c does not trigger release of cytochrome c from isolated mitochondria. Apoptosis induced by Rv1818c is blocked by the broad-spectrum caspase inhibitory peptide ZVAD-FMK. Unexpectedly, Rv1818cinduced apoptosis is not blocked in a Jurkat sub-clone deficient for caspase-8 (JI 9.2) or in cells where caspase-9 function is inhibited or expression of caspase-9 reduced by small interfering RNA (siRNA), arguing against a central role for these caspases in Rv1818c-induced apoptotic signaling. Depleting cellular pools of the mitochondrial protein Smac/DIABLO substantially reduces apoptosis consistent with mitochondrial involvement in this death pathway. We present evidence that Rv1818c-induced apoptosis is blocked by the co-transfection of an endogenous inhibitor of caspase activation XIAP (X-linked Inhibitor of Apoptosis Protein) in T cells. Taken together, these data suggest that Rv1818c-induced apoptotic signaling is likely regulated in part by the Smac-dependent activation of caspases in cells.

2178

**Autophagy Controls *Salmonella* Infection in Response to Injury to the *Salmonella*-Containing Vacuole**

C. L. Birmingham,[1,2] A. C. Smith,[1,2] M. A. Bakowski,[1,2] N. Mizushima,[3] T. Yoshimori,[4] J. H. Brumell[2,1]; [1]Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada, [2]Infection, Immunity, Injury and Repair, Hospital for Sick Children, Toronto, ON, Canada, [3]Bioregulation and Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, [4]Cell Genetics, National Institute of Genetics, Mishima, Japan

*Salmonella enterica* serovar Typhimurium is a facultative intracellular pathogen that causes disease in a variety of hosts. *S.* Typhimurium invades the cells of its host and typically occupies a membrane-bound compartment called the *Salmonella*-containing vacuole (SCV). The bacteria modify the fate of the SCV using two independent type III secretion systems (TTSSs). Autophagy is a host cell cytosolic degradation pathway recently shown to interact with intracellular pathogens in a variety of ways. Here we investigated the involvement of autophagy in *S.* Typhimurium infection. We show that a subset of intracellular *S.* Typhimurium are recognized by the autophagy system at 1 h post infection. Confocal analysis showed that the autophagic marker LC3 associated with bacteria in the cytosol as well as with bacteria in SCVs. This association was dependent upon bacterial protein synthesis and the *Salmonella* pathogenicity island (SPI)-1 TTSS. Autophagy-deficient ($atg5^{-/-}$) cells showed higher numbers of *S.* Typhimurium in the cytosol and were more permissive for intracellular replication than normal cells. However, autophagy did not recognize Δ*sifA* bacteria that had escaped from the SCV at late times in infection (8-10 h). We propose a model in which the host autophagy system recognizes cytosolic bacteria and SCVs damaged by the SPI-1 TTSS to retain intracellular *S.* Typhimurium within vacuoles early after infection.

2179

**Endotoxin and Lipotechoic Acid Inhibit Plasma Gelsolin Severing Activity**

R. Bucki, P. A. Janmey; Institute for Medicine and Engineering, University Of Pennsylvania, Philadelphia, PA

Endotoxin and Lipotechoic Acid Inhibit Plasma Gelsolin Severing Activity R. Bucki and P. A. Janmey University of Pennsylvania, Institute for Medicine and Engineering, 1010 Vagelos Research Laboratories 3340 Smith Walk Philadelphia, PA, 19104 USA. Gelsolin is the first recognized member of an extensive protein family found in eukaryotic organisms ranging from amebas to humansthat severs actin filaments in a reaction that is regulated by Ca2+ and inositol lipids. Although originally identified as a cellular cytoplasmic protein implicated in the regulation of cellular actin remodeling, gelsolin also exists as an abundant secreted isoform of nearly identical structure and a product of the same gene. The function of this protein, designated plasma gelsolin, which circulates in humans at an average concentration of 250 mg/L, is mostly unknown. Cytoplasmic gGelsolin binds with high affinity and selectivity to polyphosphoinositides and to lysophosphatidic acid (LPA), which strongly inhibits gelsolin's actin filament severing function. In this study we have used spectrofluorometric techniques to evaluated the interaction of two highly acidic bacterial lipids, endotoxin (LPS) and lipotechoiclipoteichoic acid (LTA) binding towith a rhodamine B-labeled peptide PBP10 (RhB-QRLFQVKGRR), a $PIP_2$-binding site of gelsolin. Additionally, we have evaluated the effect of LPS and LTA on gelsolin's F-actin severing activity by measuring pyrene-F-actin depolymerization rates. We found that LPS and LTA bind to PBP10 peptide. When added to gelsolin LPS and LTAand strongly inhibit gelsolin's actin severing activity. These findings indicated that bacterial compounds could affect the F-actin buffering system of blood, and suggest gelsolin involvement in LPS and LTA traffic regulation.

2180

**Role of the Molecular Chaperone Hsp90 in Folding and Processing of the Poliovirus Capsid Precursor Protein P1**

R. Geller,[1] M. Vignuzzi-Fabbri,[2] R. Andino,[2] J. Frydman[1]; [1]Biological Sciences, Stanford University, Stanford, CA, [2]Microbiology & Immunology, University of California San Francisco, San Francisco, CA

Viruses depend on the host cellular machinery for successful propagation. In particular, the folding and assembly of elaborate oligomeric viral structures such as the capsid may require assistance of the cellular folding machinery. Here we use Poliovirus as a model system to examine the role of molecular chaperones during infection. We find that the essential molecular chaperone Hsp90 is required for succesful poliovirus replication. Accordingly, treatment of tissue culture cells with the Hsp90 inhibitor Geldanamycin significantly inhibits the replication cycle of Poliovirus as well as that of the closely related Rhinovirus, the most frequent agent of the common cold. The Hsp90 chaperone system appears specifically required for folding and/or processing the capsid precursor protein P1, but not for other steps in the viral life cycle, including entry into the cell, uncoating of the capsid and viral RNA translation or replication. Strikingly, despite the rapid mutation rate of Poliovirus, we were unable to obtain viruses resistant to the Hsp90 inhibitor, indicating that the chaperone requirement cannot be easily circumvented. Our findings highlight the potential of chaperone inhibitors as antiviral agents.

2181

**Dynamic Gag Complex-Actin Interactions during Equine Infectious Anemia Virus Assembly and Budding**

C. Chen,[1] J. Jin,[1] K. M. Weixel,[2] M. Rubin,[3] J. K. Craigo,[1] D. B. Stolz,[3] S. C. Watkins,[3] O. A. Weisz,[2] R. C. Montelaro[1]; [1]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA, [2]Department of Medicine, Renal-Electrolyte Division, University of Pittsburgh School of Medicine, Pittsburgh, PA, [3]Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Retrovirus assembly and budding is a highly concerted process mediated by specific interactions between viral Gag polyproteins and host cofactors. Gag proteins recruit various components that regulate the endocytic pathway, including the formation of endosomes and multivesicular bodies for viral assembly and budding. Our previous studies have demonstrated a role for the association between Gag protein and the actin cytoskeleton in virion production of equine infectious anemia virus (EIAV), a lentivirus related to HIV-1. Here we further examined the Gag interaction with the actin cytoskeleton and its role in retrovirus assembly and budding, using high resolution imaging techniques including confocal microscopy, immuno-scanning EM, and bimolecular fluorescence complementation (BiFC) and biochemical analyses. Our confocal microscopy and immuno-scanning EM data demonstrated proximate location of EIAV Gag polyproteins with filamentous actins and BiFC analyses further confirmed the intimate interactions (2-6 nm) between actin and EIAV Gag polyprotein. Rapid migration (up to 0.2 μm/s) of Gag-actin complexes in the cytoplasm was also observed in living cells. Characterization of the viral determinants for actin interaction revealed an involvement of capsid and nucleocapsid -mediated Gag polyprotein multimerization prior to actin association and a positive correlation between actin interaction and virion production. These data indicate that oligomeric Gag proteins specifically recruit and adapt the actin filament network for trafficking and assembly of retroviral particles.

2182

**Epitope Mapping of Anti-Dengue Virus Nonstructural Protein 1 Antibodies that Cross-React to Endothelial Cell Surface**

Y. Lin, H. Cheng, S. Wan, C. Lin; Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan Republic of China

The onset of vascular leakage and hemorrhagic diathesis is one of the life-threatening complications that occur in dengue patients. The host-virus interactions that induce autoimmune responses may have implications in dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), yet the mechanisms remain unclear. Our previous studies showed a mechanism of molecular mimicry in which antibodies (Abs) directed against dengue virus (DV) nonstructural protein 1 (NS1) cross-reacted with endothelial cells and induced these cells to undergo apoptosis and immune activation. Some endothelial cell surface molecules recognized by anti-DV NS1 have been identified by 2D gel electrophoresis followed by Western blotting and mass spectrometry analysis. After alignment to database of NCBI and SwissPort, these proteins were identified as beta chain of H+-transporter/ATP synthase, protein disulfide isomerase-related protein, vimentin intermediate filament, heat shock protein 60, and protein disulfide isomerase. Furthermore, phage-displayed peptide library and peptide array of DV NS1 were used to determine the possible cross-reactive epitopes. Both data showed a conserved a.a. 315-324 peptide in C terminal region of DV NS1, called C20-10, as a cross-reactive epitope corresponding to bioinformatic prediction of EMBOSS Antigenic program. Synthetic oligopeptides of C20, a cysteine-rich region of a.a. 311-330

peptide as a speculated candidate was previously shown. This study demonstrated that C20-10 was also specifically bound by anti-DV NS1 and anti-target proteins. In contrast to normal controls and DF patients, higher titers of antibodies against C20 and C20-10 were detected in DHF patient sera. Taken together, these results suggested that anti-C20-10 may play an important role in the autoimmunity of DHF/DSS pathogenesis. In future, three-dimensional structure of DV NS1 determined by synchrontron radiation protein crystallography or NMR will be assessed for entire epitope mapping.

2183

**Tat Downregulation Protects Chronically HIV-Infected Jurkat Cells From Fas-Mediated Apoptosis**

A. J. Calderon, J. A. Lavergne; Microbiology, University of Puerto Rico - School of Medicine, San Juan, PR

AIDS is characterized by the progressive loss of both HIV-infected and uninfected $CD4^+$ T lymphocytes through apoptosis. However, the exact mechanisms and putative viral gene products involved in the regulation of apoptosis and cell cycle are not clearly defined. There is still much controversy regarding the fact that expression of HIV Tat and Vpr may induce apoptosis in some types of cells, yet may induce apoptosis resistance in others. To further evaluate the role of HIV Tat in apoptosis regulation in an *in vitro* model of chronic HIV-infection using Jurkat cells (J1.1 clone), we blocked the expression of Tat and Vpr by RNA interference (RNAi). We designed several intracellular expression vectors to create stable gene knockdown of either Tat or Vpr, and then characterized the apoptosis development and cell cycle progression in cells chronically infected with HIV. We found that RNAi against one Tat mRNA region (anti-Tat-1 vector) reduced the expression of both Tat and Vpr expression at the RNA level by almost 37%, and reduced Tat-expressing cells by 32%. In addition, viral replication in anti-Tat-1-treated cells stimulated by TNF-α, was reduced by 40%, supporting the role of Tat in HIV-1 replication. Anti-Tat-1-treated cells were found to be more resistant to nuclear apoptosis than mock-transfected J1.1 cells after stimulation with anti-Fas. These results suggest that Tat may have a role in enhancing Fas-mediated apoptosis during HIV-infection. These observations correlate with previous findings from Bartz and Emerman (1999), which found that a Tat-expressing cell line had increased expression and activity of caspase 8, an initiator of the Fas apoptosis pathway. We suggest that targeting HIV-1 Tat may be an effective means of reducing both viral replication and the progressive loss of $CD4^+$ T cells due to apoptosis in $HIV^+$ individuals.

2184

***Boehmeria Nivea* Root Extract Suppresses Supernatant Hepatitis B Virus Surface Antigen and Viral DNA in Human Hep3B Hepatoma Cells**

K. Huang; Biotechnology, National Tsing-Hua University, Hsinchu, Taiwan Republic of China

**Abstract** In Taiwan, there has been a history of using the herb *Boehmeria nivea* for medical purposes such as hepatoprotection. The aim of this report is to study the effects of *B. nivea* root extract on HBV replication in Hep3B cell model system. HBsAg and HBV DNA were measured by enzyme-linked immunosorbant assay (ELISA) and PCR, respectively. Expressivity of viral gene *preS1* was determined by reverse transcriptase-PCR (RT-PCR), and cytotoxicity, by methyl tetrazolium (MTT) assay. In addition, Lamivudine (3TC) was used as positive control for drug efficacy assays. In Hep3B cells treated with *B. nivea* extract, secretion of HBsAg into the medium was significantly suppressed and no viral DNA could be detected; however, this effect was reversible. Similar to 3TC, *B. nivea* extract was not cytotoxic towards Hep3B, CCD966SK or mouse primary liver cells; it did not affect HBV genome stability and viral gene expression. *B. nivea* extract exhibited anti-HBV activities by potently inhibiting secretions of HBsAg and levels of supernatant HBV DNA, but it conferred no cytotoxicity. According to these results, we deduce that the anti-HBV effects manifested by *B. nivea* extract were probably derived from blockage in viral nucleocapsid assembly, or reduced stability of nucleocapsids that contain pregenomic RNA.

2185

**The Molecular Cell Biology of the Immunomodulatory E3-19K Protein from Adenovirus**

M. Bouvier, H. Liu, J. Fu; School of Pharmacy, University of Connecticut, Storrs, CT

E3-19K is a type I membrane glycoprotein expressed by adenoviruses (Ads) for the purpose of modulating host antiviral immune responses. E3-19K associates with class I MHC molecules in the endoplasmic reticulum (ER) and blocks their transport to the cell surface thereby inhibiting presentation of virally-derived antigenic peptides to T cells. To date, little is known about molecular aspects of the mechanism by which E3-19K targets class I MHC molecules for retention in the ER. Towards this goal, we have developed an expression system for the ER-lumenal domain of the Ad type 2 E3-19K protein in baculovirus-infected insect cells. We have characterized the physicochemical properties of recombinant, soluble E3-19K using a variety of biophysical techniques. Results showed that the protein is monomeric in solution, thermally stable, and possesses a well-defined three-dimensional structure. In vitro reconstitution studies showed that recombinant, soluble E3-19K associates with recombinant, soluble peptide-deficient as well as peptide-filled HLA-A*1101 molecules. The complexes formed with high-affinity and in a 1:1 stoichiometry. These results demonstrate that interaction between E3-19K and class I MHC molecules occurs in the absence of any other host or Ad proteins. Our results provide the first example of a viral immunomodulatory protein that interacts with conformationally distinct forms of class I MHC molecules. Our studies also showed that peptide-deficient HLA-A*1101 molecules sequestered by E3-19K are capable of maturing into peptide-filled molecules, suggesting a role for E3-19K in maturation of class I molecules in Ad-infected cells. Together, our results suggest that Ads have evolved to exploit the early and late stages of the class I antigen presentation pathway to evade the immune system. These studies advance our understanding of the molecular cell biology underlying Ad pathogenesis. Research funded by NIH/NIAID (AI055717).

2186

**Interactions between HIV-1 and HIV-2 Gag Polyproteins *in vivo***

V. P. Boyko; HIV Drug Resistance Program, National Cancer Institute, Frederick, MD

HIV-1 and HIV-2 use the same receptor and co-receptors, thereby targeting the same cell populations in coinfected individuals. To identify potential interaction between these two human pathogens, we examined whether HIV-1 and HIV-2 Gag proteins can coassemble and functionally complement each other. We designed HIV-1- and HIV-2-based vectors with mutations in either the nucleocapsid (NC) domain or the PTAP motif of Gag; all of these mutants have severely reduced virus titers. Flow cytometry analysis revealed that coexpression of the homologous NC and

PTAP mutants allowed the coassembly and complementation of the two mutant proteins resulting in recovery of virus infectivity. Coexpression of the HIV-1 and HIV-2 gag mutants could also generate infectious viruses; furthermore, the heterologous complementation could be at similar level of efficiency as the homologous complementation. We then directly demonstrated that HIV-1 and HIV-2 Gag can interact and coassemble, using the Bimolecular Fluorescence Complementation assay (BiFC). In this assay, yellow fluorescence protein (YFP) was divided into an N- and a C-terminal portion (ny and cy) and fused to target proteins. If the target proteins interacted and brought ny and cy together, then YFP emission could be detected. We detected strong YFP emission signal not only with the homologous pairs of HIV Gag fusion proteins but also upon coexpression of heterologous pairs of Gag fusion proteins. Taken together, our results indicate that HIV-1 and HIV-2 Gag polyproteins can coassemble and complement each other during virus replication. To our knowledge, this is the first demonstration that the authentic Gag of two distinct retroviruses can coassemble and complement each other's function. These studies have important implications for the efficacy of anti-AIDS drug treatments and the evolution of primate lentiviruses.

## Cell Growth and Division (2187-2189)

2187

### Substrates for Cyclin dependent Kinases in the Promotion of Cell Cycle Transitions

T. Hunt; Cancer Research UK, Clare Hall Laboratories, Herts, United Kingdom

The key to cell cycle control is the idea of transitions between the phases of the cycle: G1 to S-phase, G2 to M-phase, the metaphase to anaphase transition and the return from mitosis to interphase. Entry to S-phase requires cyclin dependent kinase (Cdk) activity, normally provided by cyclin E-Cdk2 in the cells of higher eukaryotes, while entry into mitosis requires the combined activity of cyclins A and B together with Cdk1. We can now express active forms of all these kinases in bacteria, which allows us to investigate their substrate specificity, to explore the question of how many proteins must be phosphorylated in order to promote these transitions. And also to ask why it is, for example, that although cyclin E-Cdk2 can accelerate entry into mitosis under some circumstances, it appears to be unable to promote entry into mitosis by itself. It turns out that enumerating substrates for protein kinases is a considerable challenge, and I will report on progress in my laboratory.

2188

### Contributions Made by Cdc25 Protein Phosphatases to Cell Cycle- and Checkpoint-Control in Embryonic and Adult Cell Cycles

H. Piwnica-Worms; Department of Cell Biology and Physiology, Howard Hughes Medical Institute and Washington University School of Medicine, St. Louis, MO

The Cdc25 phosphatases positively regulate cell division by dephosphorylating and activating cyclin dependent protein kinases (Cdks). In addition, Cdc25 proteins are targets of negative regulation by the DNA damage and replication checkpoints. In humans and rodents, there are three family members - Cdc25A, -B, and -C, encoded by three genes. Murine forms of *Cdc25* exhibit distinct patterns of expression throughout development and in adult tissues. In addition, Cdc25 family members can be distinguished based on their intracellular localization, their abundance and/or activity throughout the cell cycle, the Cdks that they target for activation and whether they are overexpressed in human cancers. Furthermore, the Cdc25A- and Cdc25C-regulatory pathways are inhibited by UCN-01, a protein kinase inhibitor currently in clinical trials for cancer treatment. We are interested in defining the contributions made by individual members of the Cdc25 family to cell cycle- and checkpoint-control in mammals and in determining whether Cdc25-regulatory pathways are valid targets for the development of anti-cancer agents. With this goal in mind, targeting gene strategies were employed to disrupt *Cdc25* family members in mice. The consequences of disrupting *Cdc25A*, *Cdc25B* and *Cdc25C*, either singly or in combination, will be discussed.

2189

### Mitotic Spindle Morphogenesis: An Interphase Connection

Y. Zheng; Department of Embryology, The Carnegie Institution of Washington, Baltimore, MD

Accurate execution of mitosis is essential for equal chromosome segregation and for the maintenance of genome stability. Proper partitioning of other cellular structures and signaling molecules into daughter cells during mitosis is also important for cell fate determination and stem cell maintenance. Although cell cycle research in the past has shed light on the regulation of mitosis, our studies have revealed significant regulatory and morphological complexities of mitosis. More importantly, we have shown that the regulation of mitosis involves cellular machineries that were previously thought to have functions only in interphase. I will describe two new pathways that coordinate with the cell cycle machinery to regulate mitotic spindle morphogenesis and chromosome segregation. I will also discuss how the new pathways may influence the fate of the cell in the next cell cycle.

## Yeast Genomics in the Classroom: Using the Yeast Deletion Collection to Study Environmental Toxins and Food Additives (2190)

2190

### Yeast Genomics in the Classroom: Using the Yeast Deletion Collection to Study Environmental Toxins and Food Additives

A. C. Ballew, M. Cyert, T. Stearns; Biological Sciences, Stanford University, Stanford University, CA

The goal of the HHMI sponsored Pre-Grad Program at Stanford University is to prepare students to pursue advanced degrees in graduate school by providing coursework that both teaches the fundamentals of modern experimental science, and informs students of the exciting career opportunities. At the heart of the Pre-Grad Program is a lecture/lab course that trains undergraduate students to solve biological problems using modern tools of genetics and genomics. The goal of the class is to understand the effects of environmental toxins or food additives on *Saccharomyces cerevisiae*. To foster a more inquiry-driven learning environment, the course is focused on compounds whose effects on eukaryotic cells are not well understood. Students in the course work together to perform a genome wide screen using the yeast deletion collection and identify mutants that are hypersensitive to several compounds. Genomics and database tools are used to analyze the gene lists and students design a project to follow up on findings from one of the screens. Students are required to submit a written research proposal and give regular presentations of their data to the class. At the end of the course the findings from the projects are presented at a departmental poster session and students submit a final paper describing their results. The writing assignments and presentations teach students valuable skills about communicating science. Students reported feeling more engaged in the course because these compounds are relevant to them and because they were doing original research. Results from at least two student projects have developed into undergraduate thesis projects. We conclude this course is a good model for engaging students in original research in a classroom setting.

## Cytoskeletal Dynamics in Living Cells (2191-2196)

2191

### Endothelial Cell Migration in 3D collagen matrices replicates *in vivo* morphodynamics

R. S. Fischer; Department of Cell Biology, Scripps Research Institute, La Jolla, CA

Previous work has demonstrated that substrate compliance and topology can have dramatic effects on cell morphology, indicative of alterations in spatial adhesion cues and/or actomyosin functions. Meanwhile, a large database of information on cell protrusion and the underlying actin dynamics has been generated in cells cultured in two dimensions. To translate this knowledge into more physiological conditions, we have investigated the protrusion morphodynamics and actin turnover in endothelial cells in both traditional two dimensional culture conditions and three dimensional collagen gels, and compared these protrusive structures to those found in branching vasculature *in vivo*. In all three systems, endothelial cells protrude with similar cyclic frequencies and rates, but use remarkably different cellular structures to do so. In three dimensional collagen gels, large flat lamellipodial structures are replaced by long, brachiated pseudopods, which contain relatively stable cortical actin surrounding microtubules. The tips of these pseudopods exhibit dynamic growth cone-like protrusions and filopodia. In some individual cells, both long pseudopods and flat lamellipodia-like structures can be seen, indicating that changes in gene expression are not required for the switch from lamellar to pseudopod morphology. We further demonstrate by FRAP analyses that actin turnover in these protrusion structures displays two recovery rates which occur in spatially distinct zones. In posterior regions of the pseudopod tips, recovery rates consist of both fast, isotropic diffusional components and a slower component likely derived from filament turnover. At the very tip of pseudopod extensions, new fluorescence accumulation occurs with a single, rapid rate indicative of diffusion-limited accumulation. These data indicate that although the morphology of protrusive structures in three dimensions is dramatically different from traditional two-dimensional cell structures, similar underlying mechanisms govern the morphodynamics in both cases.

2192

### Spatio-temporal Coordination between Protrusion, Actin Network Assembly, Network Flow and Adhesion in Migrating Epithelial Cells

M. Machacek, K. Hu, C. Waterman-Storer, G. Danuser; Cell Biology, TSRI, La Jolla, CA

Protrusion of the leading edge initiates cell migration. The current understanding is that a densely branched network of actin filaments mediates the protrusion process. In epithelial cells two networks with different dynamical and molecular properties have been identified; the lamellipodium which is a 1-2μm wide band adjacent to the leading edge and the lamella which spans from the leading edge towards the cell body. The role and particularly the interaction of these two networks during protrusion are unclear. To address these questions we use quantitative Fluorescent Speckle Microscopy to measure network flow velocities, network assembly and disassembly rates and adhesion molecule accumulation as well as cell edge tracking to measure protrusion and retraction velocities with submicron resolution. The heterogeneous characteristics of the leading edge motion allowed us to observe simultaneously many states of the protrusion machinery. By statistical accumulation of the state variations we were able to identify the timing and causality between actin assembly, flow and edge movement. We found two distinct mechanisms of how protrusion and retraction are controlled by actin dynamics of the lamellipodium. The mechanisms differ in the temporal relation between protrusion, assembly, network flow and adhesion strength modulation but in both myosin II was not involved. The lamella was found to be kinematically decoupled from the lamellipodium and not directly related to protrusion. However, we measured that lamellipodium assembly was delayed by 10-20s relative to protrusion and therefore could not trigger protrusion events. We speculate that the lamella initiates protrusion and subsequently mediates lamellipodium formation which completes the protrusion cycle. This demonstrates that the two networks are mechanically and, as shown in a previous study (Ponti *etal.* Science) molecularly distinct structures, yet their dynamics are closely coordinated in mediating protrusion.

2193

### Novel Roles of Actin Binding Proteins in *Listeria monocytogenes* Actin-Based Motility Revealed Within a Cellular Context

R. Buencamino, R. D. Vale; Graduate Group in Biophysics, Cellular and Molecular Pharmacology and HHMI, University of California, San Francisco, San Francisco, CA

*Listeria monocytogenes* is a Gram-positive bacterial pathogen that hijacks the host actin cytoskeletal machinery to move within and spread directly between host cells. In order to study the role of the actin binding proteins in *Listeria* motility we infected Schneider 2 (S2) cells with *Listeria* and examined actin-based *Listeria* motility in wild-type cells and cells depleted of various actin binding proteins by RNA interference (RNAi). Using an automated tracking algorithm we focused on two main parameters: velocity and actin tail length. As expected, *Listeria* velocity was severely reduced after depletion of the monomer binding protein profilin (50% reduction) and the depolymerizing protein cofilin (50% reduction). *Listeria* in cofilin-depleted cells also exhibited discontinuous motility; this “stop-and-go” behavior may reflect a temporary accumulation of an actin shell followed by symmetry breaking. We also found unexpected defects for two protein depletions. First, the depletion of actin monomer binding protein cyclase-associated protein (CAP) caused a severe reduction in motility velocity (50% reduction), indicating an important and unappreciated role for this protein in actin-based propulsion. We also found that depletion of the actin crosslinker fascin from S2 cells caused a five-fold increase in the actin tail length and an increase in velocity by a factor of three when compared to wild-type cells. Thus, the use of in vivo motility assays in conjunction with RNAi has allowed us to uncover new roles of proteins in actin-based *Listeria* motility which have not been apparent from reconstituted assay systems.

2194

### Tau Protein's Effect On In-vitro Microtubule-based Transport: Implications For In-vivo Dynamics

S. Gross,[1] M. D. Vershinin,[1] S. J. King[2]; [1]Developmental & Cell Biology, University of California, Irvine, Irvine, CA, [2]Molecular Biology and Biochemistry, University of Missouri-Kansas City, Kansas City, MO

Aberrant functioning of the tau protein is a common feature of many neurodegenerative diseases such as the Alzheimer's disease, and tau misregulation has been linked to impaired neuronal microtubule-based transport. To better understand how tau alters transport, we mimicked some aspects of in-vivo complexity via the use of multiple motors in an in-vitro bead assay. This assay allows us to control both the number of motors per cargo and the concentration of tau on the microtubules (MTs), and to investigate how the motion of cargos changes as a result of the interaction

between these two factors. As expected, we find that cargos driven by multiple kinesin motors on bare MTs exhibit persistent motion over many microns, typically staying attached until reaching the end of the MT. By contrast, in the presence of tau on MTs, the persistence of cargos driven by multiple kinesin motors is severely depressed, and at large tau concentrations can be reduced close to the single-motor limit. However, the presence of tau on MTs does not have a commensurate effect on single-motor driven cargos. Our data suggests that tau does not simply block MT tracks, but rather reduces the number of active motors, by preventing the motors on a cargo from binding or rebinding to the MTs. We therefore propose a novel role for the tau protein: that of regulating MT-based transport by controlling the number of active motors per cargo. Such regulation could be particularly important at axonal branch points, where tau could be regulating cargo switching between MTs, and in growth cones, where local control of the number of active MT-associated motors could influence the transfer of cargos from MTs onto actin filaments.

2195

### The Formation of Antiparallel Microtubule Bundles in Fission Yeast

M. E. Janson, I. Loiodice, P. T. Tran; Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

Cells like neurons, epithelial cells, and myotubes all organize linear arrays of microtubules independent from the centrosome. A clear understanding of how these microtubules are nucleated and spatially organized is lacking. We studied the formation of 'non-centrosomal' microtubule bundles in interphase fission yeast cells. In their simplest form, these structures contain two anti parallel microtubules that are slightly overlapped at their minus ends. Bundle formation started from a single microtubule that was nucleated from a gamma-tubulin complex at the nuclear envelope. Then additional gamma-tubulin complexes bound along the side of this microtubule and nucleated a second microtubule in a direction opposite to initial growth. After this secondary nucleation the minus ends of the two microtubules did slide towards each other, which required the action of minus-end-directed kinesin-14 (klp2p). This finding demonstrates that dispersed microtubule nucleation and microtubule sliding can generate highly organized microtubule arrays. In the absence of klp2p, bundled microtubules still grew in counter opposing directions, but did not slide, suggesting that growth polarity is set by non-motor proteins. A strong candidate for this function is the conserved protein ase1p (homologues to PRC1/MAP65), which we found bundles microtubules *in vitro*. In fission yeast, ase1-GFP localized to zones of anti parallel microtubule overlap in both the spindle midzone and interphase bundles. In interphase *ase1*Δ cells, microtubule sliding was observed, but no stable zones of microtubule overlap were formed. Possibly, microtubule sliding (klp2p) needs to be counteracted by static bundling (ase1p) to achieve well-defined zones of MT overlap. Similar mechanisms may be at work in the mitotic spindle, in which sliding molecular motors and ase1p/PRC1 organize the spindle midzone.

2196

### Structural Intermediates in Microtubule Dynamics and Their Role in Cellular Function

E. Nogales; Molecular and Cell Biology Department, Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, CA

The dynamic behavior of microtubules relays on changes in the assembly properties of tubulin driven by its nucleotide state. Using cryo-electron microscopy we have analyzed the structure of stabilized intermediates in the microtubule assembly and disassembly processes. Bound to GDP the tubulin dimer is kinked and can only associate longitudinally into curved protofilaments that are unable to form functional lateral interactions. Our GDP-conformation of tubulin shows for the first time differences between the intra and inter dimer interfaces and it suggests the effect of destabilizing factors. Using a non-hydrolyzable GTP analogue we have temperature-trapped an early state in assembly that precedes microtubule closure and nucleotide hydrolysis. Our study shows that upon GTP binding the shape and association of tubulin dimers straightens enough to allow for the formation of lateral contacts in open sheets with alternating native and modified lateral interfaces in a transient polymer that subsequently rolls into a close microtubule. The existence of these structural intermediates is suggestive of a functional role in which specific cellular factors may recognize these distinctive tubulin platforms for spatial/temporal localization. Additionally, the active conversion between conformations can be used, as in the case of the bending of protofilaments during microtubule depolymerization, as a source of energy to power the movement of cellular components (e.g. the yeast kinetochore complex Dam1).

## Epithelial Morphogenesis & Polarity (2197-2202)

2197

### Mechanisms of AP-1B-dependent Targeting of Basolateral Transmembrane Proteins in Polarized Epithelial Cells

H. Fölsch; Northwestern University, Evanston, IL

Cellular asymmetry in epithelial cells manifests itself in the organization of two distinct plasma membrane domains. To maintain this intrinsic polarity, the cells have to faithfully sort transmembrane proteins to the apical or basolateral plasma membrane during biosynthetic or endocytic protein delivery. To fulfill some of these functions, epithelial cells express the clathrin adaptor complex AP-1B. In contrast to the ubiquitously expressed AP-1A, which is involved in endosomal/lysosomal targeting, AP-1B was demonstrated to play an important role in sorting of proteins such as LDL receptors and Tfn receptors to the basolateral membrane (Folsch et al., 1999). Although AP-1A and AP-1B differ only in the incorporation of their medium subunits mu1A or mu1B, respectively, both complexes were found on distinct vesicle populations (Folsch et al., 2003). Furthermore, AP-1B but not AP-1A was found to play a role in the membrane recruitment of subunits of the exocyst complex needed for tethering basolateral vesicles to the fusion site. Interestingly we found that the C-terminus of mu1B is sufficient for Exo70 recruitment. This explains on a molecular level the differential behavior of the closely related AP-1A and AP-1B complexes in exocyst recruitment.

2198

### Septins Regulate Golgi-to-Plasma Membrane Traffic in Polarizing Epithelia

E. T. Spiliotis,[1] S. Hunt,[1] V. Varenika,[1] M. Kinoshita,[2] W. J. Nelson[1]; [1]Molecular and Cellular Physiology, Stanford University, Stanford, CA, [2]Biochemistry and Cell Biology Unit, Kyoto University, Kyoto, Japan

Septins are conserved GTP-binding proteins that associate with cellular membranes, and the actin and microtubule cytoskeleton. In budding yeast, septins are required for polarized membrane growth, but septin function in polarized epithelial cells is unknown. In non-polarized Madin-Darby canine kidney (MDCK) cells, septin 2 (Sept2) was found at sites of membrane fission from the trans-Golgi network. By time-lapse fluorescence

microscopy, CFP-tagged apical and basal-lateral marker proteins exited the trans-Golgi network in tubular membrane carriers that co-aligned with Sept2YFP. Endogenous Sept2 also colocalized with Golgi membrane tubules after treatment with BFA, an inhibitor of membrane fission. Sept2YFP and Sept6GFP were also observed to move along curve-linear pathways with instantaneous velocities (0.4+/-0.2 micrometers/second) resembling those of *bona fide* vesicle markers (eg. VSV-G-GFP); this was consistent with Sept2 presence on purified Golgi vesicles. Moreover, Sept2YFP and Sept6GFP accumulated with E-cadherin-dsRed-labeled membranes at nascent sites of cell-cell contact, which contain the exocyst complex and are presumed to be hot spots of vesicle fusion. To probe for septin function(s) in the establishment of epithelial cell polarity, non-polarized MDCK cells were treated with mock and Sept2 siRNAs for 48 hrs and then plated onto polycarbonate filters at a confluent cell density for 24 hours. In Sept2-depleted cells, development of the lateral membrane domain was impaired. Lateral membranes appeared short and the apical junctional complex marker protein ZO-1 mislocalized to the lower half of the lateral membrane. Significantly, the basal-lateral marker protein VSV-G-YFP accumulated in a post-Golgi intermediate compartment and recruitment of the exocyst complex to adherens junctions was down-regulated. Hence, Sept2 is required for membrane delivery to the growing lateral domain of polarizing cells. Overall, we suggest that septins regulate Golgi-to-plasma membrane traffic by coupling membrane fission/fusion to cytoskeleton-based mechanisms of vesicle transport.

2199

**A Novel Molecular Mechanism by Which PAR-1 Regulates the Epithelial Apico-basal Polarity**

M. Hirata, A. Suzuki, Y. Amano, N. Sakai, K. Shimada, S. Ohno; Molecular Biology, Yokohama City University School of Medicine, Yokohama, Japan

aPKC/PAR system serves as the evolutionarily-conserved, ubiquitous molecular machinery which amplifies various cell polarity signals and links them to the corresponding global cell polarization processes. The system includes three Ser/Thr kinases, aPKC, PAR-1, and LKB1, all of them have been shown to play crucial roles for the development of epithelial polarity. We have shown that aPKC is indispensable for the establishment of the epithelia-specific apical junctional complex (AJC) including adherens junction (AJ) and tight junction (TJ), while one of the mammalian PAR-1 variants, PAR-1b, is essential for the development of the membrane domains which mainly occurs after the AJC establishment. We also found that, at TJ, aPKC suppresses the invasion of basolaterally-localized PAR-1b into the apical membrane in cooperation with PAR-5/14-3-3, and thereby maintains a balance between the apical and lateral membranes. In this work, we report the identification of a membrane-skeletal protein that specifically interacts with PAR-1b. They are asymmetrically co-localized at the basolateral membrane of polarized MDCK cells, and co-precipitated from these cells. RNAi knockdown of this membrane-skeletal protein in MDCK cells resulted in the same polarity defects as PAR-1b showing impaired membrane domain development with compromised cortical actin staining. Unexpectedly, the membrane-skeletal protein is not required for the basolateral localization of PAR-1b, but rather, PAR-1b kinase activity is essential for the basolateral localization of this membrane-skeletal protein. Several lines of evidence suggested that PAR-1b promotes the assembly of extracellular matrix by way of the interaction with this protein, and thereby affects the lateral membrane extension and establishment of membrane polarity. These results provide a novel mechanism by which PAR protein regulates apico-basal polarity of epithelial cells.

2200

**Endocytic control of epithelial polarity and proliferation**

T. Vaccari, D. Bilder; Department of Molecular/Cell Biology, University of California, Berkeley, Berkeley, CA

Acquisition of appropriate cellular architecture is required not only for tissue morphogenesis but also permits proper cell-cell communication. A dramatic example of the coordination between cell architecture and cell signaling is seen in Drosophila 'neoplastic' tumors, wherein loss of epithelial polarity is coupled to an inability to control cell proliferation. To investigate the links between epithelial organization and growth-control signaling, we have carried out a mosaic genetic screen to isolate new mutations that cause neoplastic or malignant tumors in the fly. Through this screen we have identified a number of components that regulate intracellular vesicle traffic. Surprisingly, the largest group of proteins identified act not in the exocytic but the endocytic pathway, revealing a critical role for endocytic traffic in homeostatic control of both epithelial polarity and proliferation. While the polarity phenotypes of many of the endocytic mutants are similar, the effects on cell signaling and cell proliferation can be dramatically different. We will discuss the functions of both known and new members of the endocytic pathway, and how blocks at discrete steps of endocytic traffic alter the activity of specific signaling pathways.

2201

**Hexagonal Packing of Drosophila Wing Epithelial Cells by the Planar Cell Polarity Pathway**

A. Classen,[1] K. I. Anderson,[2] E. Marois,[1] S. Eaton[1]; [1]Max-Planck-Insitute of Molecular Cell Biology and Genetics, Dresden, Germany, [2]Beatson Institute for Cancer Research, Glasgow, United Kingdom

The mechanisms that order cellular packing geometry are critical for the functioning of many tissues, but are poorly understood. Here we investigate this problem in the developing wing of Drosophila. The surface of the wing is decorated by hexagonally packed hairs that are uniformly oriented. They are constructed by a hexagonal array of wing epithelial cells. Wing epithelial cells are irregularly arranged throughout most of development but become hexagonally packed shortly before hair formation. During the process, individual cell boundaries grow and shrink resulting in local neighbour exchanges, and E-cadherin is actively recycled. Successful hexagonal repacking depends on the endocytic recycling pathway, dynamin and on the activity of the planar cell polarity (PCP) proteins. Interfering with Rab11 and dynamin function leads to loss of E-cadherin from epithelial contacts at the time of hexagonal repacking. Epithlial disintegration caused by the lack of dynamin is enhanced by PCP mutants. PCP mutants fail to efficiently assemble boundaries with new neighbours and do not distribute their junctional material symmetrically. We propose that planar cell polarity proteins influence Dynamin-dependent recycling of adhesive components, and suggest that this is a common mechanism for their action in diverse systems.

2202

**Polycystin-1, STAT6 and P100 Function in a Novel Pathway that Transduces Ciliary Mechanosensation to Gene Expression and is Activated in Polycystic Kidney Disease**

S. H. Low,[1] S. Vasanth,[2] C. H. Larson,[3] S. Mukherjee,[4] N. Sharma,[5] M. T. Kinter,[3] M. E. Kane,[6] T. Obara,[6] T. Weimbs[7]; [1]Neuroscience Research

Institute, University of California Santa Barbara, Santa Barbara, CA, [2]School of Biomedical Sciences- Cleveland Clinic, Kent State University, Kent, OH, [3]Cell Biology, Cleveland Clinic, Cleveland, OH, [4]Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA, [5]Biological Geological and Environmental Science, Cleveland State University, Cleveland, OH, [6]Department of Medicine, MetroHealth Medical Center, Case Western Reserve University, Cleveland, OH, [7]Department of Molecular, Cellular, and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA

Primary cilia are implicated in the pathogenesis of autosomal-dominant polycystic kidney disease (ADPKD) which results from defects in polycystin-1 (PC1) but the function of PC1 and mechanism underlying cyst formation remain poorly understood. Here we show that PC1 participates in a novel signaling pathway that controls the transduction of a mechanical signal from primary cilia to changes of gene expression. PC1 undergoes proteolytic cleavage that results in nuclear translocation of its cytoplasmic tail. Exogenous expression of the human PC1 tail results in renal cyst formation in zebrafish embryos. The PC1 tail interacts with the transcription factor STAT6 and the co-activator P100, and stimulates STAT6-dependent gene expression. Under normal conditions STAT6 and P100 localize to primary cilia of renal epithelial cells. Cessation of apical fluid flow results in nuclear translocation of STAT6. Cyst-lining epithelial cells in ADPKD exhibit highly elevated levels of nuclear STAT6, P100 and the PC1 tail. These results identify a novel mechanism of cilia function and show that this pathway is inappropriately activated in ADPKD.

## Lipid-Mediated Signals (2203-2208)

2203

### FGF-induced Vesicular Release of Sonic Hedgehog and Retinoic Acid in Leftward Nodal Flow is Critical for Left−Right Determination

Y. Tanaka, Y. Okada, N. Hirokawa; Department of Cell Biology and Anatomy, University of Tokyo Graduate School of Medicine, Tokyo, Japan

The precise specification of left−right asymmetry is an essential process for patterning internal organs in vertebrates. In mouse embryonic development, the symmetry-breaking process in left−right determination is initiated by a leftward extraembryonic fluid flow on the surface of the ventral node. However, it is not known whether the signal transduction mechanism of this flow is chemical or mechanical. Here we show that fibroblast growth factor (FGF) signalling triggers secretion of membrane-sheathed objects 0.3−5 μm in diameter termed 'nodal vesicular parcels' (NVPs) that carry Sonic hedgehog and retinoic acid. These NVPs are transported leftward by the fluid flow and eventually fragment close to the left wall of the ventral node. The silencing effects of the FGF-receptor inhibitor SU5402 on NVP secretion and on a downstream rise in $Ca^{2+}$ were sufficiently reversed by exogenous Sonic hedgehog peptide or retinoic acid, suggesting that FGF-triggered surface accumulation of cargo morphogens may be essential for launching NVPs. Thus, we propose that NVP flow is a new mode of extracellular transport that forms a left−right gradient of morphogens.

2204

### Sphingosine-1-phosphate Lyase Promotes Cell Cycle Arrest and Apoptosis Through P53 and P38 Dependent Mechanisms

B. Oskouian, P. Sooriyakumaran, M. A. Lee, J. D. Saba; Children's Hospital Oakland Research Institute, Oakland, CA

Sphingosine-1-phosphate (S1P) is a bioactive lipid with diverse effects on cell fate and function including growth promotion, organismal development, and cell differentiation and death. S1P lyase is the enzyme responsible for irreversible degradation of S1P. Our laboratory is exploring the role that S1P lyase plays in modulating the effects of S1P in cells and organisms. Previously, we reported that overexpression of S1P lyase renders HEK293 cells hypersensitive to serum deprivation by promoting apoptosis. We have recently found that S1P lyase expression enhances apoptotic responses to cytotoxic agents, including the topoisomerase II inhibitor etoposide. Changes in cell cycle progression after etoposide treatment were also markedly different in cells overexpressing S1P lyase, which accumulated in $G_2$/M as determined by flow cytometry and by increased expression of the mitotic marker S10 phosphohistone H3. We have investigated potential mechanisms by which S1P lyase affects these cellular responses to etoposide. Cell cycle changes and enhanced apoptosis in S1P lyase expressing cells were both abrogated by inhibition of p53 function through chemical and molecular approaches, suggesting that both phenomena occur through p53-dependent mechanisms. In contrast, inhibition of p38 MAPK activity with SB203580 reversed the increased apoptosis but not the cell cycle changes in S1P lyase expressing cells treated with etoposide, suggesting that S1P lyase may affect cell cycle and apoptotic responses to stress through divergent regulatory pathways. Importantly, S1P lyase gene expression was found to be upregulated in response to variety of stressful stimuli, including DNA damage. These findings indicate that S1P lyase may play a crucial role in mediating the cellular responses to DNA damage and other stressful conditions.

2205

### Differential Requirements for Sphingosine Kinase Isoenzymes in Cell Migration Toward EGF

S. Spiegel,[1] N. C. Hait,[1] S. Sarkar,[1] A. Mikami,[1] M. Maceyka,[1] S. Milstien[2]; [1]Department of Biochemistry, Virginia Commonwealth University, Richmond, VA, [2]Laboratory of Cellular and Molecular Regulation, NIMH, Bethesda, MD

Sphingosine-1-phosphate (S1P), produced by two sphingosine kinase isoenzymes, SphK1 and SphK2, is the ligand for a family of five specific G protein-coupled receptors that regulate cytoskeletal rearrangements and cell motility. Whereas many growth factors stimulate SphK1, much less is known of the regulation of SphK2. We have now found that similar to PDGF, EGF transiently stimulates SphK1. Importantly, however, EGF also stimulates SphK2. This is the first example of an agonist-dependent regulation of SphK2. Chemotaxis of HEK 293 cells toward EGF was inhibited by N,N-dimethylspingosine, a competitive inhibitor of both SphKs, implicating S1P generation in this process. Overexpression of SphK1 in HEK 293 cells enhanced EGF-mediated cell migration, while expression of SphK2 had no significant effect. Furthermore, catalytically inactive SphK1, but not inactive SphK2, blocked EGF-induced migration of these cells. Moreover, downregulating expression of SphK1 in HEK 293 cells with a specific siRNA also abrogated migration toward EGF, while decreasing SphK2 expression had no effect. Surprisingly, however, in MDA-MB-453 breast cancer cells, downregulation of either SphK1 or SphK2 abrogated migration toward EGF. Collectively, our results suggest that both SphK1 and SphK2 play important roles in migration of MDA-MB-453 cells toward EGF.

2206

### OSBP is a Cholesterol-Regulated Scaffolding Protein in Control of ERK1/2 Activation

P. Wang, J. Weng, R. G. W. Anderson; Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX

OSBP is a Cholesterol-Regulated Scaffolding Protein in Control of ERK1/2 Activation Ping-yuan Wang, Jian Weng and Richard G. W. Anderson Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas. TX 75390-9039 Oxysterol binding protein (OSBP) is the founding member of a family of sterol binding proteins implicated in vesicle transport, lipid metabolism and signal transduction. Here OSBP was found to function as a cholesterol-binding scaffolding protein coordinating the activity of two phosphatases to control the extracellular signal-regulated kinase (ERK) signaling pathway. Cytosolic OSBP formed a ~440 kDa oligomer with a member of the PTPPBS family of tyrosine phosphatases, the serine/threonine phosphatase PP2A, and cholesterol. This oligomer had dual specific phosphatase activity for pERK. These two enzymes each depend on the activity of the other to coordinately remove phosphate from both the threonine and the tyrosine residues of pERK. When cell cholesterol was lowered, the oligomer disassembled and the level of pERK rose. The oligomer also disassembled when exposed to oxysterols. Interestingly, it was reported that OSBP moves from cytosol to the Golgi apparatus under both of the conditions. We found that OSBP binds cholesterol and oxysterol at two different sites. Increasing the amount of OSBP oligomer rendered cells resistant to the effects of cholesterol depletion and decreased the basal level of pERK. In contrast, decreasing the amount of OSBP oligomer by RNA interference of OSBP mRNA caused a sustained activation of ERK1/2. Thus, cholesterol functions through its interaction with OSBP outside of membranes to regulate the assembly of an oligomeric phosphatase that controls a key signaling pathway in the cell.

2207

### Diacylglycerol Incorporation upon Compartment Mixing acts as an Upstream Signal for Actin Assembly during Compensatory Endocytosis in *Xenopus* Eggs

H. E. Yu,[1] W. Bement[2]; [1]Program in Cellular and Molecular Biology, University of Wisconsin -- Madison, Madison, WI, [2]Zoology Department, University of Wisconsin -- Madison, Madison, WI

Exocytosis of cortical granules (CGs) in *Xenopus* eggs is followed by direct membrane retrieval, resulting in compensatory endocytosis. The compartments thus formed become surrounded by actin “coats”, which are generated by Cdc42-dependent actin polymerization (Sokac *et al.*, Nat Cell Biol. 5: 727-32, 2003). Here, we assessed the role of conventional protein kinase Cβ (PKCβ) in the formation of actin coats by using time-lapse confocal microscopy on eggs expressing different fluorescently labeled proteins, in combination with inositol triphosphate ($IP_3$) uncaging to elevate $Ca^{2+}$ and trigger CG exocytosis. Perturbation of PKCβ perturbed actin coat assembly, as well as Cdc42 activation, showing that PKCβ signals via Cdc42 to initiate actin coat assembly. Importantly, incorporation of diacylglycerol (DAG) from the plasma membrane (PM) into the CG membrane during CG-PM fusion sustained PKCβ recruitment on exocytosing CGs, indicating that DAG incorporation is key to actin coat assembly. A parallel study on PKCη revealed that this novel PKC had a different recruitment pattern and when perturbed, did not affect actin coat assembly, but perturbed cortical actin integrity, demonstrating that PKCη plays a different role during exo/endocytosis of CGs. Thus, while DAG incorporation during CG-PM compartment mixing is crucial for PKCβ activation to initiate actin assembly, the interplay between PKCβ and PKCη is also necessary for proper exo/endocytosis of CGs.

2208

### The Role of the Phosphoinositides at the Golgi Complex

M. A. De Matteis,[1] A. Godi,[1] A. Di Campli,[1] G. D’Angelo,[1] E. Polishchuk,[1] R. Polishchuk,[1] D. Alessi,[2] J. M. Lucocq,[3] J. Saus[4]; [1]Cell Biology/Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy, [2]Division of Cell Signalling, University of Dundee, Dundee, United Kingdom, [3]MSI/WTB Complex, University of Dundee, Dundee, United Kingdom, [4]Molecular Pathology, Instituto de Investigaciones Citologicas, Valencia, Spain

The phosphoinositides (PIs) act as precursors for second messengers and as membrane ligands for protein modules. Specific lipid kinases and phosphatases are differentially located and regulated in individual cell organelles, thus generating non-uniform distributions of the PIs. At the Golgi complex, the PIs act as key regulators of anterograde membrane traffic via PtdIns(4)P, and of the structure of the organelle via PtdIns(4,5)P2. We have investigated this system using a combination of approaches that range from knock-out of the Golgi-localized PI kinases and phosphatases (by pharmacologically means, or by dominant-negative mutants and siRNAs), through “sequestering” of PtdIns(4)P using PtdIns(4)P-binding domains, to direct interference with the localization/ levels of the PtdIns(4)P targets. We thus show that PtdIns(4)P has a pivotal role at the TGN, and specifically at the crossroads between the endocytic and exocytic pathways. Here, the PtdIns(4)P-generating enzymes and the PtdIns(4)P-binding proteins (such as the FAPPs, OSBP1 and CERT) regulate the transport of cargoes that are destined for the plasma membrane. Finally, we provide evidence that in conjunction with the small GTPase ARF, PtdIns(4)P may also act as a cue for the orienting/ directing of the non-vesicular transfer of lipids from the endoplasmic reticulum to the Golgi complex, and to specific Golgi subcompartments.

## The Membrane Cytoskeleton (2209-2214)

2209

### Ankyrin-G and Assembly of Specialized Membrane Domains

K. Kizhatil,[1] P. J. Mohler,[2] W. Yoon,[1] V. Bennett[1]; [1]Cell Biology, Duke University Medical Center/HHMI, Durham, NC, [2]Pathology, Vanderbilt University, Nashville, TN

The ability of cells to segregate proteins within specialized membrane domains underlies much of vertebrate physiology. For example, generation of action potentials by neurons and heart cells requires excitable membranes. Similarly, transcellular transport by epithelial tissues such as kidney and intestine requires distinct apical and lateral membranes. A central question is whether common mechanisms drive assembly of specialized membranes in these diverse cells. In support of this idea, ankyrin-G, a versatile membrane adaptor, is required for restriction of components of excitable membranes of both neurons and heart as well as formation of lateral membranes of epithelial cells. We have used siRNA to deplete 190 kDa ankyrin-G and beta-2 spectrin in cultured human bronchial epithelial cells. Cells depleted of either protein convert to a squamous morphology, with nearly complete loss of lateral membrane, while the total membrane surface area and apical polarity are preserved. Moreover depletion of either ankyrin-G or beta-2 spectrin also abolishes biogenesis of the lateral membrane during cytokinesis. Mutations of ankyrin-G that abolish spectrin-binding activity also eliminate activity in restoring lateral membranes of ankyrin-G-depleted cells. Ankyrin-G therefore requires beta-2 spectrin for its function in assembly of the lateral membrane. Ankyrin-G and beta-2 spectrin colocalize at the lateral membrane. However, beta-2-

spectrin also is localized separately from ankyrin-G in an intracellular tubulovesicular compartment that is enhanced by Brefeldin A and is distinct from conventional Golgi or ER. Together, these results identify beta-2-spectrin and ankyrin-G as essential participants in a conserved pathway for assembly of the lateral membrane domain. Given roles of ankyrin-G in neurons and heart cells, the ankyrin-spectrin pathway may have a more general function in assembly of diverse membrane domains.

2210

**The Presynaptic Spectrin Skeleton is Essential for Synapse Stabilization**

J. Pielage,[1] R. D. Fetter,[2] G. W. Davis[1]; [1]Department of Biochemistry, University of California, San Francisco, San Francisco, CA, [2]Laboratory of Neural Circuits and Behavior, The Rockefeller University, New York, NY

Precise neural circuitry is established and maintained through a regulated balance of synapse stabilization and disassembly. Currently, little is known about the molecular mechanisms that specify synapse stability versus disassembly. The spectrin-based membrane skeleton has long been considered a candidate for participating in both synapse organization and maintenance. Spectrin is a major component of pre- and postsynaptic assemblies throughout the mammalian and invertebrate nervous systems. We demonstrate that presynaptic spectrin is an essential scaffold that is required to maintain synapse stability at the *Drosophila* neuromuscular junction (NMJ). Loss of presynaptic spectrin leads to synapse disassembly and ultimately to the elimination of the NMJ. Synapse elimination is documented through light-level, ultrastructural, and electrophysiological assays. These combined assays reveal that impaired neurotransmission is secondary to synapse retraction. We demonstrate that loss of presynaptic, but not postsynaptic, spectrin leads to the disorganization and elimination of essential synaptic cell-adhesion molecules. In addition, we provide evidence of altered axonal transport and disrupted synaptic microtubules as events that contribute to synapse retraction in animals lacking presynaptic spectrin. Our data suggest that presynaptic spectrin functions as an essential presynaptic scaffold that may link synaptic cell adhesion with the stabilization of the underlying microtubule cytoskeleton. We will present further data towards the molecules and mechanisms that link the spectrin skeleton to synaptic cell adhesion molecules and to the underlying cytoskeleton. We will also present data that postsynaptic spectrin is required to maintain the postsynaptic density and glutamate receptor clusters.

2211

**Myoferlin Mediates Myoblast Fusion**

K. R. Doherty,[1] A. T. Cave,[2] D. B. Davis,[2] A. J. Delmonte,[2] A. Posey,[2] J. U. Earley,[2] M. Hadhazy,[2] E. McNally[2]; [1]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL, [2]Department of Medicine, University of Chicago, Chicago, IL

Muscle growth occurs during embryonic development and continues in adult life as regeneration. During embryonic muscle growth and the regenerative growth of mature muscle, singly nucleated myoblasts fuse to each other to form myotubes. These processes initiate with cell-cell recognition followed by the alignment and fusion of two apposed lipid bilayers. The large size of mature myofibers and their constant exposure to deformation through muscle contraction necessitates a mechanism for membrane repair in muscle. Dysferlin, the product of the Limb Girdle Muscular Dystrophy type 2 locus, is a membrane-associated protein that is necessary for efficient, calcium-sensitive membrane resealing in muscle (Bansal et al. Nature 2003). The loss of dysferlin leads to an accumulation of submembraneous vesicles. We now show that the related protein, myoferlin, is highly expressed in myoblasts undergoing fusion and that myoferlin is expressed at the site of myoblasts fusing to myotubes. Like dysferlin, we found that the first C2 domain of myoferlin binds phosphotidylserine/phosphotidylcholine in a calcium-sensitive manner. We generated mice with a null allele of myoferlin, and found that myoferlin null myoblasts form large myotubes less efficiently in vitro, consistent with a defect in the fusion of myoblasts to myotubes. In vivo, myoferlin null mice have smaller muscles than littermate controls, and myoferlin null muscle has a marked reduction in the largest diameter myofibers. We also found that expression of myoferlin is greatly increased in acutely injured muscles. Consistent with a role for myoferlin in myoblast-mediated muscle regeneration, myoferlin null muscle regenerates less well than wildtype muscle and instead displays a dystrophic phenotype. These data support a role for myoferlin in the maturation of myotubes and the formation of large myotubes that arise from the fusion of myoblasts to multinucleate myotubes.

2212

**Components Required for Membrane Deposition at the Site of Scission in *C elegans* Embryos**

J. M. Squirrell,[1] K. J. C. Verbrugghe,[2] H. Zhang,[2] J. G. White[1]; [1]Laboratory for Molecular Biology, University of Wisconsin-Madison, Madison, WI, [2]Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI

Previous studies showed that secretion is necessary for the final stage of cytokinesis (i.e. scission) in *C. elegans* (Skop et al.,Curr Biol 2001). We used the plasma membrane probe FM 2-10, in conjunction with multiphoton microscopy, to observe the movements of the plasma membrane and endocytosed vesicles during cytokinesis of *C. elegans* embryos. In wild-type embryos, membrane accumulates at the apex of cleavage furrow shortly following the completion of furrow ingression. This accumulation is altered or absent in embryos depleted for centralspindlin components, all of which are required for scission. In addition, embryos depleted for CAR-1, a protein that localizes to cytoplasmic puncta and is homologous to RNA associated proteins, have abnormal spindle dynamics and no spindle midzone microtubules, lack membrane accumulation at the apex of the late cleavage furrow and fail in scission. In *car-1(RNAi)* embryos, ZEN-4, a member of the centralspindlin complex, is found on the ingressing furrow, a localization that has previously been shown to be sufficient for cytokinesis in the absence of a spindle midzone (Verbrugghe and White, 2004), indicating that in the absence of CAR-1 the furrow localization of ZEN-4 is not sufficient for cytokinesis to complete. *car-1(RNAi)* embryos also show defects in the structure of the endoplasmic reticulum and in RAB-11 distribution. This suggests that endomembrane organization, in conjunction with the spindle midzone microtubules and centralspindlin proteins, is an important component of the mechanisms used in cytokinesis.

2213

**Single Particle Tracking of Murine Polyomavirus-like Particles on Live Cells and Artificial Membranes**

H. Ewers,[1] A. E. Smith,[1] I. F. Sbalzarini,[2] H. Lilie,[3] P. Koumoutsakos,[2] A. Helenius[1]; [1]Institute of Biochemistry, ETH, Zurich, Switzerland, [2]Institute of Computational Science, ETH, Zurich, Switzerland, [3]Institute of Biotechnology, Martin-Luther-University in Halle, Zurich, Switzerland

The lateral mobility of fluorescently labeled murine polyomavirus-like particles (VLPs) bound to live cells and artificial lipid-bilayers was studied by single particle tracking using total internal reflection fluorescence (TIRF) microscopy. The particle trajectories were analyzed according to their

diffusion rates and their modes of motion as described by the moment scaling spectrum. We found that VLPs bound to their ganglioside receptor in lipid bilayers exhibited free diffusion. In contrast, trajectories on live 3T6 mouse fibroblasts revealed three distinct modes of mobility. These were rapid random motion, confined movement to small zones of 30-60 nm in diameter, and confined movement in such zones with a slow drift. After binding to the cell surface, the particles typically underwent free diffusion for 5-10 s before they became confined in an actin filament-dependent manner. Neither clathrin-coated pits nor caveolae were involved in the confinement of the VLPs. While depletion of cholesterol dramatically reduced mobility of VLPs independently of actin, inhibition of tyrosine kinases had no effect on confinement. The results suggested that clustering of ganglioside molecules by the multivalent VLPs induced transmembrane-coupling in lipid rafts that led to confinement of the virus/receptor complex by cortical actin filaments.Single particle tracking provides a powerful tool to analyze the sequence of events during initial virus-host cell interactions.

2214

**Membrane Deforming Properties and Interaction With Dynamin of a Family of Actin Regulatory Proteins That Contain an F-Bar Domain**

T. Itoh,[1] A. Roux,[1] B. Habermann,[2] H. Werner,[1] K. S. Erdmann,[1] P. De Camilli[1]; [1]Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Scionics Computer Innovation, c/o Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

Membrane trafficking and cell motility require the deformation of cell membranes. Deformations are achieved both by forces extrinsic to the membrane as well as by structural modifications in the bilayer or at the bilayer surface that favor the acquisition of curvature. BAR domain proteins, for example, bind to membranes and induce or sense (and therefore stabilize) membrane curvature. Via studies in living cells and in cell-free models involving purified proteins and artificial lipid membranes, we have demonstrated that a family of proteins (including Toca-1, FBP17, CIP4, Pacsin1) previously known to function as key regulators of actin nucleation have in addition powerful lipid bilayer deforming properties. Their membrane deforming activity was mapped to an N-terminal module (F-BAR) which shows sequence similarity to the BAR domain and includes the previously defined FCH module. Moreover, several of these proteins bind dynamin, a GTPase implicated both in endocytosis and actin dynamics. Overexpression of the Toca-family proteins CIP4 and FBP17 in COS7 cells induces tubular invaginations of the plasma membrane. In addition CIP4 and FBP17, but not deletion constructs of these proteins lacking the SH3 domain, recruit dynamin to these invaginations. The properties of BAR and F-BAR domain proteins to induce tubular invaginations of the plasma membrane are further enhanced by disruption of the actin cytoskeleton using Latrunculin B. These results suggest a very close interplay between the mechanisms that control actin nucleation and those which mediate plasma membrane deformations underlying endocytosis.

## Neuronal Polarity & Axo-Dendritic Growth (2215-2220)

2215

**Neuronal Polarity and Cytoskeleton**

T. Yoshimura, K. Kaibuchi; Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, Nagoya, Japan

The neuron is one of the most highly polarized cells and is comprised of two structurally and functionally distinct parts, an axon and dendrites. In cultured hippocampal neurons, neurons extend several minor processes during the first 12-24 h after plating. Then, one of the processes begins to extend rapidly to form an axon, resulting in the morphological polarization of the neuron. The remaining processes result in the morphological features of dendrites. Previously, we showed that collapsin response mediator protein-2 (CRMP-2) is enriched in the growing axon of hippocampal neurons, the overexpression of CRMP-2 induces the formation of multiple axons and elongation of the primary axon, and truncated fragment of CRMP-2 or knockdown of CRMP-2 by RNAi inhibits axon formation. Thus, CRMP-2 appears to play a crucial role in the determination of axon/dendrite fate and axon elongation. We have recently found that CRMP-2 interacts with tubulin heterodimers to promote microtubule assembly for axon growth, and that CRMP-2 binds to Sra-1, an effector of Rac1 to regulate WAVE-dependent reorganization of actin filaments. CRMP-2 links Kinesin-1 to tubulin heterodimers and Sra-1, and mediates the Kinesin-1-dependent transport of tubulin heterodimers and the Sra-1/WAVE complex to developing axons. We have also found that the PAR-6/PAR-3 complex and the Akt/GSK-3β pathway are involved in neuronal polarization downstream of PI3-kinase. The PAR-6/PAR-3 complex mediates the Cdc42-induced Rac1 activation through direct interaction with STEF/Tiam1 (Rac GEF) for axon growth. GSK-3β phosphorylates CRMP-2 and inactivates its tubulin-binding activity. Akt appears to phosphorylate GSK-3β and inactivates its kinase activity, thereby increasing non-phosphorylated active CRMP-2, which promotes axon outgrowth. This time, we summarize and discuss functions of these ‘polarity’ molecules in regulation of neuronal polarity and cytoskeleton.

2216

**Ena/VASP Proteins are Required for Neuritogenesis in Cortical Neurons**

E. W. Dent, A. Kwiatkowski, D. Rubinson, L. Mebane, U. Philippar, M. Barzik, E. M. Kovacs, F. B. Gertler; Biology, MIT, Cambridge, MA

Genetic studies in worms, flies and mice have implicated Ena/VASP proteins in major guidance decisions in developing central nervous system (CNS) neurons. In an accompanying abstract (Kwiatkowski et al.) we describe the phenotype of mice in which all three mammalian Ena/VASP family members (Mena/VASP/EVL) have been deleted. Surprisingly, these mice exhibit very little, if any, axonal growth from the cortical plate in developing neocortex, implicating Ena/VASP proteins in neuritogenesis. We have cultured cortical neurons from triple knockout mice to determine the mechanism by which Ena/VASP family proteins regulates neuritogenesis. Mena/VASP/EVL triple knockout (mmvvee) cortical neurons attach and spread a circular lamellipodia like wild type controls (stage 1). However, the lamellipodia is devoid of filopodia and bundled actin filaments and over 80% of the neurons fail to extend any minor processes (stage 2), even after 48hrs in culture. Nevertheless, they are differentiated neurons because they express the neuron specific BIII-tubulin. Transfection of low levels of either Mena, VASP or EVL can rescue this phenotype, allowing the neurons to extend filopodia, minor processes and subsequently a single axon (stage 3). Addition of laminin, but not fibronectin or collagen, rescues this stage 1 phenotype as well by forming loosely bundled actin filaments in filopodia-like protrusions. Addition of blebbistatin, a specific myosin II inhibitor, also induces neurite extension rather rapidly from mmvvee cortical neurons, apparently by inhibiting retrograde actin flow and compaction of the peripheral F-actin meshwork in the transition zone. The inhibition of actin retrograde flow allows microtubules to extend to the periphery, promoting process extension. These data support a new function for Ena/VASP proteins in neuritogenesis and indicate that Ena/VASP dependent bundled actin filaments in filopodia of stage 1 cortical neurons are necessary for neurite extension.

2217

**AFAP: Regulating the Transition**

L. M. Lanier; Department of Neuroscience, University of Minnesota, Minneapolis, MN

Growth cone motility and neurite branching are dependent on the coordinated regulation of the actin and microtubule cytoskeletons. The peripheral region of the growth cone is dominated by actin-rich filopodia, while the central region contains abundant microtubules and a sparse meshwork of actin filaments. The transition region is a dense actin network that separates the central and peripheral regions and appears to serve as a boundary to microtubule advance. Studies have shown that the stochastic ability of microtubules to cross through the transition region and explore the peripheral region is a key determinant of growth cone turning. While proteins in the central and peripheral region have been identified, little is known about the protein(s) that regulate actin dynamics in the transition region. Here we report that the Actin Filament Associated Protein (AFAP110/120) is specifically enriched in the growth cone transition region. AFAP110, the ubiquitous form of AFAP, was previously identified as a tyrosine phosphorylated protein in Src kinase transformed cells. AFAP110 binds actin filaments, co-localizes with actin stress fibers in fibroblasts and is important for the stretch induced activation of Src. We demonstrate that AFAP120, the neuronal-specific form, is one of the most abundant tyrosine phosphorylation substrates in developing neurons and that Protein kinase C modulates this phosphorylation in a Src kinase dependent manner. The fact that AFAP is a stretch responsive, Src-regulated actin-binding protein in the transition region of the growth cone suggests that it could be involved in regulating actin dynamics and, indirectly, microtubule advance, during growth cone turning and branching. Indeed, preliminary experiments indicate that RNAi knockdown of AFAP alters neurite outgrowth and branching.

2218

**Cdc42 Bioprobes Reveal Signal Integrations within a Single Neuron Undergoing Dendrogenesis**

D. Kamiyama, A. Chiba; Cell and Developmental Biology, University of Illinois, Urbana, IL

Cdc42 is a highly conserved and versatile cytosolic Rho GTPase. In neurons, multiple signaling pathways are thought to converge and diverge at Cdc42 during differentiation. However, little is known about how Cdc42 activities are spatiotemporally regulated and integrated to specific signaling molecules within individual neurons. Here, we show that genetic deletion/disruption of Cdc42 in the *Drosophila* pioneer motoneurons results in their failure to initiate dendrogenesis. Cdc42 is found ubiquitously within the neurons. However, GFP-tagged and/or FRET-based *in vivo* bioprobes reveal that both activation of Cdc42 and localization of its effectors are each regulated dynamically within the neurons. Remarkably, the two display a spatiotemporal coincidence at the onset of dendrogenesis. Further analysis suggests that, whereas Robo receptor is required for activating Cdc42 with a specific temporal restriction, Dscam is responsible for spatially limiting a CRIB-containing effector responsible for initiating dendrogenesis within the neurons. Altogether, our study reveals dynamic spatiotemporal integrations of Cdc42 signaling within a single differentiating neuron *in vivo*.

2219

**Cytoplasmic Dynein Transports Short Microtubules, Aligns Long Microtubules, and Opposes Retraction of the Axon**

K. A. Myers,[1] Y. He,[1] F. Francis,[2] M. M. Black,[2] P. W. Baas[1]; [1]Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, [2]Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA

We recently reported that cytoplasmic dynein transports short microtubules down the axon. It seems reasonable to posit that the longer immobile microtubules are subjected to these same forces, and hence that cytoplasmic dynein plays a broader role in the organization and disposition of the axonal microtubule array. To investigate this, we determined the effects of suppressing dynein functions in cultured rat sympathetic neurons either by depletion of dynein heavy chain using siRNA or by disruption of dynactin via overexpression of P50-dynamitin. Both methods reduced the anterograde transport of short microtubules in the axon by about half. Axonal growth was fairly normal in both cases, except when the neurons were re-plated after a few days of treatment. After re-plating, axonal growth was stunted, with microtubules appearing dramatically curved and misaligned, particularly in their distal regions. This effect was more profound in the dynein-depleted neurons. When the neurons were treated with donors of nitric oxide, the axons of dynein-depleted neurons showed a marked increase in the degree of retraction, and again, the microtubules were dramatically curved and misaligned. This was not observed in the neurons overexpressing P50-dynamitin, but is reminiscent of earlier studies in which axonal retraction occurred in response to microinjection of higher levels of P50-dynamitin. Taken together, these results are consistent with a model whereby cytoplasmic dynein not only transports short microtubules, but also serves to align the longer microtubules and offset other forces that tend to compress the microtubules and cause the axon to retract.

2220

**Mechanisms of Developmental Axon Pruning**

E. D. Hoopfer, A. Penton, R. Watts, L. Luo; Howard Hughes Medical Institute and Department of Biological Sciences, Stanford University, Stanford, CA

Pruning of exuberant axons and dendrites is a widespread mechanism in vertebrates and invertebrates for achieving the mature pattern of neuronal connections. Axon pruning may also occur in response to injury or disease. In *Drosophila*, mushroom body (MB) gamma neurons undergo developmental pruning of their axonal projections in response to the cell-autonomous action of the steroid hormone ecdysone. Using these neurons as a model for axon pruning we have shown that MB axons are pruned through local degeneration of axon branches, and that this process requires the intrinsic activity of the ubiquitin-proteasome system. In addition, through the use of a genetically encoded electron microscopic (EM) marker to selectively label neuronal or glial populations in the MB, we find that degenerating axon fragments are engulfed by glia for degradation via the endosomal-lysosomal pathway, suggesting an extrinsic role for glia in axon pruning. What are the molecular mechanisms that regulate developmental pruning of axons? Recently, we conducted two screens to identify new genes involved in developmental axon pruning of MB neurons: (1) a systematic gain-of-function screen in MB neurons, and (2) a microarray screen to identify genes differentially regulated by ecdysone signaling. Both screens independently identified *boule*, a gene encoding an RNA binding protein, as being involved in axon pruning. We find that *boule* expression is rapidly down-regulated by ecdysone signaling at the onset of axon pruning and that overexpression of Boule is sufficient to inhibit axon pruning. Together these studies suggest that Boule is a critical regulator of developmental axon pruning. We also address the broader question of the molecular relatedness between axon pruning during development and after axon injury.

## Protein Folding & Quality Control (2221-2226)

2221

### Genome-wide Screens Uncover the Essential Cellular Functions and Substrates of the Hsp90 Molecular Chaperone

A. J. McClellan,[1] B. Y. Xia,[2] A. M. Deutschbauer,[3] R. Davis,[3] M. Gerstein,[2] J. Frydman[1]; [1]Biological Sciences, Stanford University, Stanford, CA, [2]Molecular Biophysics & Biochemistry, Yale University, New Haven, CT, [3]Biochemistry, Stanford University, Stanford, CA

Molecular chaperones are essential mediators of cellular folding. Yet, little is known about their respective contributions to folding in the eukaryotic cell. The molecular chaperone Hsp90 is ubiquitous throughout eukaryotes and comprises 1-2% of total cytosolic protein. Hsp90 is required for viability, even under non-stress conditions. However, only a handful of confirmed folding substrates for the Hsp90 chaperone have been identified. In order to uncover the scope of requisite cellular functions and substrates of Hsp90, we have conducted whole-genome screens in yeast using an Hsp90-inhibiting drug and the available yeast deletion collections. Data from the homozygous deletion pool experiments allowed the construction of an in-depth Hsp90 protein interaction network. Interestingly, analysis of Hsp90 function conducted at both 30 °C and 37 °C revealed that, while some deletion strains exhibited a dependence on Hsp90 for viability at both temperatures, some functional processes were uniquely affected at only one of these temperatures. These data suggest that Hsp90 carries out distinct cellular functions at normal and elevated growth temperatures. Surprisingly, a role for Hsp90 in various avenues of cellular transport was uncovered at 30 °C, while at 37 °C a dependence on Hsp90 for aspects of signal transduction, cell cycle, meiosis and cytokinesis was observed. As many essential proteins may be Hsp90 “clients”, genomic screens were also conducted using pooled heterozygous deletion strains. Numerous potential Hsp90 substrates have thus been identified and ongoing biological and bioinformatic analyses, in combination with the homozygous data, will permit us to build a complete picture of Hsp90 function in yeast. The high degree of conservation between yeast and mammalian Hsp90 enables the translation of these new data to humans, allowing for a better understanding and treatment of human diseases linked to Hsp90-dependent targets.

2222

### Role for Chaperones in Recognizing Substrates for ER Associated Degradation (ERAD), Demonstrated by *in vitro* Reconstitution of Ste6p* Ubiquitination

K. Nakatsukasa,[1] G. Huyer,[2] S. Michaelis,[2] J. L. Brodsky[1]; [1]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, [2]Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

Endoplasmic reticulum associated degradation (ERAD) clears the secretory pathway of aberrant or misfolded proteins, and these proteins are ultimately degraded by the ubiquitin-proteasome system. Ubiquitination of aberrant proteins provides a signal for their delivery to, and degradation by, the proteasome. While many components involved in the ubiquitination and proteasomal delivery of ERAD substrates have been identified, little is known about how ERAD substrates are selected for ubiquitination, especially in the case of misfolded integral membrane proteins. We have developed Ste6p*, a mutant form of Ste6p, as a model ERAD substrate in yeast. Ste6p is a multispanning membrane protein with 12 transmembrane spans and is a member of the ATP-binding cassette superfamily that also includes the cystic fibrosis transmembrane conductance regulator (CFTR). In yeast, ERAD of Ste6p* depends on Ubc6/7p (ubiquitin E2 enzymes), Doa10p (an ER integral membrane ubiquitin E3 ligase), and cytosolic molecular chaperones (the Hsp70, Ssa1p and two Hsp40s, Ydj1p and Hlj1p). To define how Ste6p* is recognized and targeted for degradation, we have reconstituted Ste6p* ubiquitination *in vitro* using microsomes and cytosol prepared from yeast. Upon incubation of Ste6p*-containing microsomes with cytosol and ATP, Ste6p* is poly-ubiquitinated, and ubiquitination is diminished when the ubiquitination machinery (Ubc6/7p and Doa10p) is mutated. Importantly, mutation of the chaperones Ssa1p or Ydj1p/Hlj1p also reduces ubiquitination of Ste6p*, suggesting that these chaperones are involved in recognizing Ste6p* for ERAD. Similar requirements are observed for the poly-ubiquitination of CFTR *in vitro*. Experiments are currently underway to define other recognition factors that act upstream of the ubiquitination machinery. The poly-ubiquitinated Ste6p* also provides a starting material for studying how misfolded integral membrane proteins are degraded by the proteasome.

2223

### ER Export and Misfolding Disease - A New View of Protein Secretion

R. L. Wiseman,[1] Y. Sekijima,[1] J. Matteson,[2] J. W. Kelly,[3] W. E. Balch[4]; [1]Department of Chemistry, The Scripps Research Institute, La Jolla, CA, [2]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [3]Department of Chemistry and The Skaggs Institute of Chemical Biology, The Scripps Research Institute, La Jolla, CA, [4]Department of Cell Biology and The Institute for Childhood and Neglected Diseases, The Scripps Research Institute, La Jolla, CA

A number of human diseases, including the familial transthyretin (TTR) amyloidoses, are associated with the misfolding and aggregation of destabilized protein variants outside the cell. Using a combined biophysical and cell biological approach, we demonstrate that both secretion through the exocytic pathway and amyloidogenicity of TTR variants correlate with the global protein energetics - defined by the kinetic and thermodynamic stability of the protein fold. These results show that proteins significantly destabilized relative to the wild-type benchmark can be secreted from the cell with full efficiency. Based on these results, we propose a new view of protein export from the ER that builds on the initial concept of quality control (QC). Instead of treating ER export as a QC test of protein functionality, we propose that each cargo protein utilizes an ER export mechanism that can be more correctly defined by the competitive interactions between the components comprising ER-assisted folding (ERAF), ER-associated degradation (ERAD), and ER-mediated export (ERME) pathways. By moving beyond the inherent limitations of QC focused on protein functionality, a new energetic based model of ER export is proposed where cargo partitioning between the ERAF, ERAD, and ERME pathways dictates export thresholds imposed by the local cellular environment. This mechanistic approach allows for a better appreciation of the general operating principles of the ER in the context of cellular physiology, protein misfolding diseases, and eukaryotic evolution.

2224

### A New Role for Ire1 in the Unfolded Protein Response in *Drosophila* Cells

J. Hollien, J. S. Weissman; Cell. and Molecular Pharm., UCSF, San Francisco, CA

The unfolded protein response (UPR), originally identified as a stress response associated with the accumulation of unfolded proteins in the endoplasmic reticulum (ER), plays an important role in the development of metazoans and in differentiation of cells specializing in secretion. A

key sensor in this response is Ire1, an ER transmembrane protein that participates in the splicing and activation of the transcription factor XBP1. To better understand the role of Ire1 and XBP1 in the UPR, we have individually depleted the mRNAs encoding these proteins in *Drosophila* S2 cells, and measured the effects on global UPR-regulated gene expression using DNA microarrays. We find that although Ire1 and XBP1 have similar effects on genes that are activated by the UPR, Ire1 additionally regulates a subset of mRNAs that are repressed by the UPR. This repression is independent of XBP1 and is not mediated transcriptionally, indicating a new role for Ire1 in regulating mRNA decay. The targets of this repression are enriched for membrane protein-encoding mRNAs, suggesting that this effect may alleviate stress by reducing the load on the ER. We are currently determining the *cis*-requirements for targets of this effect and exploring the possibility that Ire1 may directly cleave target mRNAs.

2225

**Unfolded Protein Response**

P. Walter; Department of Biochemistry/Biophysics, HHMI/UCSF, San Francisco, CA

The protein folding capacity of the endoplasmic reticulum (ER) is regulated by the unfolded protein response (UPR). Induction of the UPR leads to transcriptional upregulation of genes, including those encoding ER resident enzymes and chaperones, phospholipid biosynthetic enzymes, components involved in ER associated protein degradation, and components acting downstream of the ER in the secretory pathway. Together, these changes affect about 5% of the genes in yeast and lead to a restructuring of the secretory pathway according to the need of the cell. Studies of the UPR have revealed many surprising new ways by which eukaryotic gene expression can be controlled, including non-conventional cytoplasmic mRNA splicing, translational control by an mRNA intron, and the use of a protein kinase module as an intramolecular conformational switch. Many of the salient features of the UPR are conserved from yeast to mammalian cells.

2226

**The Yeast Unfolded Protein Response (UPR) Sensor Ire1 uses Three Functional Domains to Transmit an Unfolded Protein Signal across the Endoplasmic Reticulum Membrane**

F. R. Papa,[1,2] J. Credle,[3] P. Walter[4,3]; [1]Medicine, UCSF, San Francisco, CA, [2]California Institute for Quantitative Biomedical Research (QB3), UCSF, San Francisco, CA, [3]Howard Hughes Medical Institute (HHMI), UCSF, San Francisco, CA, [4]Biochemistry and Biophysics, UCSF, San Francisco, CA

Unfolded proteins in the endoplasmic reticulum (ER) activate the ER transmembrane protein Ire1 to trigger an adaptive pathway called the unfolded protein response (UPR). Ire1 transmits this unfolded protein signal using three functional domains: (1) an ER lumenal unfolded protein-sensing domain, and on the cytosolic side of the ER membrane (2) a protein kinase and (3) an endoribonuclease that initiates nonconventional splicing of *HAC1* messenger RNA, leading to production of the Hac1 transcription factor whose downstream targets expand ER protein-folding capacity. To refine our understanding of the structural and functional relationships between Ire1's three domains in this unique signal transduction pathway, we used mutational analyses and overexpression methods to perturb signaling by Ire1. We learned that the ER lumenal domain, whose crystal structure has been recently solved, likely uses a central cavity created by dimerization of two monomers to recognize unfolded proteins. Recognition of unfolded proteins by the ER lumenal domain leads to oligomerization of Ire1 in the plane of the ER membrane, causing trans-autophosphorylation by the kinase activity and inducing the endoribonuclease activity. Furthermore, Ire1's kinase domain requires occupancy of its active site with an adenosine nucleotide ligand to throw a conformational switch which activates the endoribonuclease; this conformational change can be mimicked with an ATP-competitive drug in drug-sensitized Ire1 mutants. Together these results suggest that unfolded proteins may cause clustering of Ire1 at the ER membrane, followed by conformational rearrangement(s) of the kinase and endoribonuclease that allow propagation of the unfolded protein signal to the nucleus.

## RNA Silencing Mechanisms (2227-2232)

2227

**Two Novel Proteins, Dos1 and Dos2 Regulate Heterochromatic RNA Interference and Histone Modification**

F. Li,[1] D. Goto,[2] M. Zaratiegui,[2] X. Tang,[1] R. Martienssen,[2] W. Z. Cande[1]; [1]Department of Molecular & Cell Biology, University of California, Berkeley, Berkeley, CA, [2]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

In fission yeast, chromosomal behavior such as sister chromatid cohesion during mitosis, and telomere clustering during meiosis, depends in part on heterochromatic modifications such as histone H3 lysine-9 di-methylation (H3mK9). These modifications are also required for gene silencing via Swi6, a homolog of Heterochromatin Protein 1 (HP-1), which recognizes Histone H3 dimethyl-lysine 9 (H3K9me2), silences transcription and retains cohesin at the pericentromeric repeats. H3K9 is methylated by the methyltransferase Clr4, which depends on RNA interference of transcripts derived from centromeric repeats. Rik1, a WD-40 repeat-containing protein, acts in a complex with Clr4 to promote H3K9 di-methylation but the mechanism underlying this interaction is poorly understood. Using a cytological screen we have identified two novel genes, *dos1*$^{+}$ and *dos2*$^{+}$, which are required for localization of Swi6. Deletions of these two genes results in mitotic and meiotic chromosome missegregation and defects in mitotic centromere and meiotic telomere clustering, as well as loss of heterochromatic silencing. Dos1 is predominantly located in the nucleus in a Dos2-dependent manner, and forms a complex with Rik1 that recruits Clr4. Each of these genes is required for the association of H3K9me2 with centromeric repeats, as well as for the production of small interfering RNA. Our findings suggest a mechanism for the recruitment of Clr4 in the RNAi-dependent heterochromatin pathway, in which Dos1 and Dos2 play essential roles.

2228

**Targets and Functions of Plant MicroRNAs**

B. Bartel,[1] A. C. Mallory,[2] D. V. Dugas,[1] D. P. Bartel[3,4]; [1]Department of Biochemistry and Cell Biology, Rice University, Houston, TX, [2]Whitehead Institute for Biomedical Research, Whitehead Institute, Cambridge, MA, [3]Whitehead Institute for Biomedical Research, Cambridge, MA, [4]Biology, Massachusetts Institute of Technology, Cambridge, MA

MicroRNAs are ~21-nt riboregulators that direct cleavage of mRNAs or attenuate productive translation. Plant microRNAs have a strong propensity to direct the regulation of transcription factors involved in cell fate determination, but the extent to which microRNAs control

development is only beginning to be understood. We have uncovered some of the developmental roles of microRNAs in the Arabidopsis model system. By overexpressing microRNAs and expressing microRNA-resistant target genes, we have exposed a posttranscriptional layer of gene regulation for several gene families and have assigned developmental and molecular roles to some of the microRNA:target mRNA relationships. Some of the developmental defects resemble phenotypes previously observed in plants expressing viral suppressors of RNA silencing and plants with mutations in genes important for microRNA biogenesis or function, providing molecular rationales for phenotypes previously associated with more general disruptions of miRNA function.

2229

### A Gene With Two Tudor Domains Regulates RNA-related Events in *C elegans*

N. Le Bot, J. Ahringer; The Gurdon Institute, Cambridge, United Kingdom

Generation of asymmetry in *Caenorhabditis elegans* embryo establishes the anterio- posterior axis of the animal. The microtubule cytoskeleton plays an important role in the asymmetric localisation of conserved polarity proteins to anterior or posterior cortical domains. These proteins in turn control the positioning of the first cleavage through regulation of microtubule function. To identify new genes required for these early events, we carried out a genome-wide RNA mediated interference (RNAi) screen in *C.elegans*. *F22D6.6* is a gene whose inactivation results in the misplacement of the first cleavage. *F22D6.6* codes for a protein of unknown function containing two Tudor domains, which are thought to mediate protein-protein interaction. These domains are found in proteins involved in RNA processing such as TSN, the micrococcal nuclease of RISC, a component of the RNAi machinery. Interestingly, *F22D6.6* was also identified in a genome wide screen looking for new components required for RNAi using an engineered RNAi sensor strain of *C.elegans (1).* RNAi related mechanims are known to mediate a variety of phenomena such as heterochromatin formation, silencing of transposable elements or developmental patterning. Two studies have also found that the silencing of a germline specific transgene is impaired by *F22D6.6(RNAi)(1, 2).* Analysis of *F22D6.6(RNAi)* oocytes and embryos showed that their cytoskeleton is abnormal. Several proteins required for normal microtubule function, like the centrosome associated protein TAC-1 are up-regulated. We are now examining the RNA levels of these proteins in *F22D6.6(RNAi)* animals as well as the distribution of different histone modifications in their germlines. The results of these studies will allow us to assess if this gene controls gene expression by regulating transcription. 1. Kim, J.K., et al., Science, 2005, 308:1164-1167. 2. Robert, V.J.P. et al., Genes and Development, 2005, 19:782-787.

2230

### Muscle Specific MicroRNAs miR-206,-1 and -133 Promote Muscle Differentiation by Post-transcriptional Downregulation of Targets by mRNA Degradation

A. Dutta, H. K. Kim, Y. S. Lee, U. Sivaprasad; Biochemistry, University of Virginia, Charlottesville, VA

The muscle specific miRNAs, miR-206, -1, and -133 are induced during differentiation of C2C12 myoblasts into myotubes in vitro. We show that differentiation from myoblast to myotube is accelerated by the introduction of these miRNAs. This is accompanied by the direct down-regulation of some but not all computationally predicted targets of the microRNAs: Connexin 43, Bind-1 and Mmd. We identify DNA polymerase alpha as a direct target and show that downregulation of the polymerase and of DNA synthesis precede the G1 arrest and cdk2 inhibition seen upon microRNA transfection. Conversely, depletion of the miRNAs by 2'-O-methyl oligonucleotide inhibits differentiation of C2C12 myoblasts and allows them to persist in active DNA replication. This is accompanied by a decrease in MHC positive cells and failure of the few MHC positive cells to elongate into long spindle shaped cells as in normal differentiation. For one of the direct targets, Bind-1, we demonstrate that the downregulation is at the post-transcriptional level and is dependent on sites that match the first 8 nucleotides (but not the entire 22 nucleotides) of the miRNA. The downregulation is however not effected by mRNA cleavage at the match site. Instead RACE-PCR identifies degradation products with multiple ends that are not restricted to the miRNA recognition sequences. This pattern differs from an siRNA-like cleavage mechanism and is consistent with the microRNAs recruiting the target to a different degradation machinery that either cleaves the mRNA at multiple sites or promotes the progressive digestion of the mRNA with several pause sites. Our results suggest that muscle-specific miRNAs promote skeletal myogenesis through direct down-regulation of many target genes by a machinery that differs from the siRNA like cleavage mechanism.

2231

### Disassembly of GW Bodies Disrupts Mammalian RNA Interference

A. Jakymiw,[1] S. Lian,[1] T. Eystathioy,[2] M. Satoh,[3] J. C. Hamel,[1] M. J. Fritzler,[2] E. K. L. Chan[1]; [1]Oral Biology, University of Florida, Gainesville, FL, [2]Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada, [3]Medicine, University of Florida, Gainesville, FL

The human GW182 protein is characterized by multiple glycine(G)/tryptophan(W) repeats and a classical RNA recognition motif. GW182 was initially demonstrated to associate with a specific subset of messenger RNAs (mRNAs) and reside within discrete cytoplasmic foci named GW bodies (GWBs), which are enriched in certain proteins involved in mRNA degradation. GWBs were identified as unique electron dense cytoplasmic structures of 100-300 nm diameter, which vary in number and size during the cell cycle; and are enlarged and more abundant in proliferating cells. *In vitro* gene knockdown of GW182 has demonstrated that GW182 functions as a matrix or scaffold, on which GWBs and their components are assembled. Recent reports have shown that exogenously introduced hAgo2 are also enriched in GWBs suggesting that RNA interference (RNAi) function may be linked to these structures. We demonstrate that endogenous hAgo2 and transfected Cy3-labeled small interfering RNAs (siRNAs) and microRNAs (miRNAs) are also present within these cytoplasmic bodies and that the GW182 protein interacts with hAgo2. Furthermore, during the process of analyzing the structural domains of GW182 and hAgo2, our laboratory noted that the N-terminal 1/3 fragment of GW182, as well as the C-terminal 1/2 fragment of hAgo2 upon transient transfection into HeLa cells disrupted endogenous GWB formation. Disruption of these cytoplasmic foci in HeLa cells, using these dominant interfering constructs impaired the silencing capability of siRNA specific to lamin A/C. *In vitro* gene knockdown of GW182 similarly disrupted GWBs and impaired RNAi functionality, thus independently verifying the results observed with the dominant interfering constructs. Our data support a model whereby the GW182 protein and/or the microenvironment of the cytoplasmic GWBs contributes to the RNA-induced silencing complex (RISC) and RNA silencing activity.

2232

### RNAi, MicroRNAs, and Human Disease

S. Hammond; Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC

MicroRNAs are short, noncoding RNAs that post-transcriptionally regulate gene expression. Over 200 microRNA genes have been identified in humans, however the specific cellular pathways they regulate are largely unknown. We have developed tools to study microRNA function in mammalian systems. We have identified a cluster of microRNAs, miR-17-92, that is overexpressed in a wide range of tumor derived cell lines. Ectopic expression of these microRNAs in a mouse model for lymphoma accelerates tumorigenesis. We have identified cell cycle and apoptotic mRNA targets of these oncogenic microRNAs. We are characterizing the biological pathways these microRNAs regulate in the context of tumorigenesis.

## Stem Cell Niches (2233-2238)

2233

### The *C elegans* Distal Tip Cell Niche, its Development and Function

N. Lam,[1] B. E. Thompson,[2] S. L. Crittenden,[3] J. Kimble[1,3,2]; [1]Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, [2]Program in Cellular and Molecular Biology, University of Wisconsin-Madison, Madison, WI, [3]Howard Hughes Medical Institute, Madison, WI

The *C. elegans* distal tip cell (DTC) is a well-defined and simple niche that controls germline stem cells. We will describe our current understanding of how the DTC is specified and how it functions to maintain stem cells. The DTC is specified, at least in part, by Wnt signaling: no DTCs are produced in mutants lacking Wnt pathway components, including POP-1/TCF and SYS-1/β-catenin; moreover, extra DTCs are made when SYS-1/β-catenin is expressed ectopically. We have identified a homeodomain-encoding gene that controls the generation of DTCs and is a direct target of Wnt/MAPK signaling. We used in vitro, tissue culture and transgenic reporter experiments to show that POP-1/TCF and SYS-1/β-catenin act directly to control the gene, and we used genetic and transgenic experiments to show that this homeodomain transcription factor is both necessary and sufficient for DTC specification. The DTC caps the end of the germline "mitotic region". BrdU experiments demonstrate that mitotic region cells are capable of self-renewal and contribute to the pool of differentiating gametes. The DTC promotes mitotic divisions by Notch signaling and a downstream network of RNA regulators. We previously showed that FBF/Pumilio is essential for maintenance of adult germline stem cells. We now find that FOG-1/CPEB acts in a dose-dependent manner to promote mitoses at a low dose and specify the sperm fate at a high dose. FOG-1/CPEB is kept low near the DTC, and increases as germ cells enter meiosis, a spatial distribution established by FBF/Pumilio. The DTC, Notch signaling and RNA regulators provide a plastic circuit to control number of mitotic cells and number of sperm.

2234

### A Rap-GEF/Rap GTPase Signaling Controls Stem Cell Niche Formation through Regulating Adherens Junctions in the *Drosophila* Testis

S. X. Hou, H. Wang, S. Singh, Z. Zheng, S. Oh, X. Chen; Laboratory of Immunobiology, National Cancer Institute, Frederick, MD

Stem cells will undergo self-renewal to produce new stem cells if they are maintained in their niches. The regulatory mechanisms that recruit and maintain stem cells in their niches are not well understood. In *Drosophila* testes, a group of 12 nondividing somatic cells, called the hub, identify the stem cell niche by producing the growth factor Unpaired (Upd). Here we show that a Rap-GEF/Rap signaling controls stem cell anchoring to the niche through regulating DE-cadherin-mediated cell adhesion. Loss of function of a *Drosophila* Rap-GEF (Gef26) results in loss of both germline and somatic stem cells. The stem cell loss phenotype of *Gef26* mutation can be enhanced by the *Drosophila Rap* mutations. Gef26 and Rap1 are enriched at the interface between the hub and stem cells. The *Gef26* mutation specifically impairs adherens junctions at the hub-stem cell interface, which results in the stem cells "walking away" from the niche and losing stem cell identity. The Rap-GEF/Rap signaling may identify an extrinsic signal that regulates the polarized niche formation for asymmetric stem cell division.

2235

### Differential Segregation of Mother and Daughter Centrosomes During Asymmetric Stem Cell Division in the Drosophila Male Germ Line

Y. M. Yamashita, M. T. Fuller; Developmental Biology, Stanford University, Stanford, CA

Stem cells are the source of highly-differentiated but short lived cells such as blood, skin and sperm. The critical balance between stem cell and differentiated cell populations are crucial for long-term maintenance of functional organs. Stem cells maintain this balance by choosing two alternative fates: stem cell self-renewal or commitment to differentiation. The *Drosophila* male germ line stem cells (GSCs) divide asymmetrically, giving rise to one stem cell and one gonialblast that initiates differentiation, thus keeping balance between stem cell and differentiating cell populations. In adult male testes, 8-10 germ line stem cells surround the somatic apical hub. The hub functions as stem cell niche by secreting a signalling ligand, unpaired (Upd), which activates the JAK-STAT pathway in GSCs to specify stem cell identity. In this signalling microenvironment, GSC orients its mitotic spindle perpendicular to the hub such that one daughter cell remains attached to the hub while the other is displaced away from the hub, thereby ensuring the asymmetric outcome of the division. We have shown that stereotyped positioning of the centrosomes during interphase plays crucial role in orienting GSCs toward the hub cells: One centrosome is always positioned close to the hub, while the other centrosome migrates toward the opposite side of the nucleus during interphase, thereby setting up the perpendicular orientation of the mitotic spindle. We found that it is always that mother centrosome stays close to the hub, while the daughter migrates. This suggests that the difference(s) between mother and daughter centrosomes may be functionally exploited to achieve the asymmetric outcome of the stem cell division.

2236

### Different Types of Niches Regulate Epithelial and Germline Stem Cells in *Drosophila*

B. Ohlstein, E. Decotto, T. Kai, T. Nystul, A. Spradling; Department of Embryology, HHMI/Carnegie Institution of Washington, Baltimore, MD

*Drosophila* germline stem cells (GSCs) at the tip of each ovariole, and at the apex of the testis, are maintained and regulated by neighboring stromal cells that generate an essential microenvironment known as a stem cell "niche." A small number of non-dividing, differentiated cap cells in the female or hub cells in the male, play critical roles by adhering to the stem cells, and by providing GSCs with local signals that control their

growth and development. Recently, we have identified and begun to analyze three types of epithelial stem cells and their niches. Ovarian escort stem cells strongly resemble the cyst progenitor stem cells of the testis, and interact with both stromal cap cells and GSCs. Adult intestinal stem cells (ISCs) maintain multiple cell types in the adult gut that strongly resemble mammalian gut lineages. ISCs contact basement membrane and mature tissue cells, but not a specialized niche cell. We have also been unable to find a stromal niche cell analogous to the cap cell in the vicinity of the ovarian follicle stem cell (SSC). Our studies argue that tissues contain multiple types of stem cell niches with distinctive regulatory regimes.

2237

**The Mammary Stem Cell Niche Modulates the Repertoire of Testicular Stem Cells**

C. A. Boulanger, D. L. Mack, B. W. Booth, G. H. Smith; MBTL, NCI/NIH, Bethesda, MD

Evidence for somatic stem cell niches has been clearly defined in several biological systems. To determine if a stem cell niche occurs in the mouse mammary gland and if it displays tissue-specific dominance, we mixed wild type mammary epithelial cells with testicular cells isolated from a WAP-Cre/R26R male donor at a 1:1 ratio and inoculated them into the cleared inguinal fat pads of immune-compromised Nu/Nu female hosts The host mice were bred 6-8 weeks later and examined 20-30 days post involution. This approach allowed for growth of mammary tissue, transient activation of the WAP-Cre gene, recombination and constitutive expression of Lac Z from the Rosa 26 promoter. Whole mount analysis was done on the inguinal glands from the injected mice. In all, 50% (n=15/30) were successful and produced mammary epithelial outgrowths. Of these 80%(n=12/15) contained blue cells. PCR analysis of the DNA from outgrowths containing blue cells confirmed the presence of both transgenes and Y chromosome-specific alleles. No LacZ-positive cells were observed in epithelial outgrowths collected from non-breeding hosts (n=6/10) indicating that pregnancy was essential for activation. The presence of male cells in significant numbers throughout mammary epithelial outgrowths suggests the following scenario: mammary epithelial cells recombine to assemble growth-competent cellular microenvironments, occasionally chimeras are formed which include male cells; development proceeds. The male cells not only contribute significantly to mammary epithelial growth but also differentiate during pregnancy activating the WAP-Cre gene and concomitantly the LacZ reporter. Both casein-positive luminal cells and myoepithelial cells were LacZ-positive within the secretory alveoli. We conclude that uncommitted testicular cells capable of self-renewal and multiple cell fates, enter mammary epithelium-specific niches and adopt the function of similarly endowed mammary cells. These results imply the tissue-dominance of stem cell niches.

2238

**Blood Island Formation in the Murine Yolk Sac**

M. J. Ferkowicz, M. C. Yoder; Pediatrics, Indiana University School fo Medicine, Indianapolis, IN

Yolk sac blood islands are the first site of hematopoiesis and vascular development in the vertebrate embryo and the first hematopoietic stem cell niche to form. The mesoderm-derived hematopoietic and endothelial lineages emerge from a common precursor, the hemangioblast and require yolk sac visceral endoderm-derived factors for blood island formation. As commonly described, undifferentiated mesodermal cells migrate into the blood island region of the yolk sac and proliferate into mesodermal masses nestled between the outer visceral endoderm and inner mesothelial cell layers. Blood islands arise as mesodermal masses differentiate into a primitive erythroblast core surrounded by endothelium. The objective of this study was to examine blood island formation in the murine yolk sac using defined cell lineage marking. FACS sorting and primitive erythroid colony forming assays reveal that primitive erythroblast progenitors are uniquely $CD41^+$ ($\alpha_{IIb}$ integrin). Flk-1 (fetal liver kinase, VEGFR-2, kdr) and VE-Cadherin (CD144) are expressed on the entire endothelial lineage including hemangioblasts. Confocal microscopy of staged murine embryos (E7.0-E8.5) reveals that committed $CD41^+$ primitive erythroblast and Flk-$1^+$/VE-Cadherin$^+$ endothelial progenitors arise near the primitive streak and subsequently migrate into the blood island region. Blood islands begin as a single circumferential band of primitive erythroblast progenitors in the blood island region of the yolk sac that completely lacks an endothelial covering. The sparse endothelial progenitors of the blood island region initially reside between the blood band and the overlying visceral endoderm. Within 6-8 hours of the onset of circulation, endothelial cells circumscribe and subdivide the blood band into a series of blood filled vascular channels. These data indicate that a new paradigm of murine blood island formation is necessary to understand the formation of this early stem cell niche.

## Signal Transduction III (2239-2265)

2239

**Functional Studies of SUMO-1 in Stress Responses, Reactive Oxygen Species Generation and Cell Death**

J. Lee, H. Hong, K. Lee; Center for Cell Signaling Research, Division of Molecular Life Sciences and College of Pharmacy, Ewha Womans University, Seoul, Republic of Korea

Small ubiquitin-like modifier (SUMO) is a member of the superfamily of ubiquitin-like polypeptides that become covalently attached to various intracellular target proteins as a way to alter their function, location, and/or half-life. Even though SUMO is the structurally related to ubiquitin molecule for protein degradation, its biological functions are distinct to ubiquitin, yet poorly understood regulatory signal. Protein ubiquitinations accumulated by various signals including cytokine treatments and cellular stresses (hypoxia and heat shock etc.), induce protein degradation in proteasome. Simultaneously, cellular accumulations of polyubiquitinated proteins by cellular stresses can induce the cell death. In this study, we examined whether protein sumoylation counteracts to the protein ubiquitination. We found that the overexpression of SUMO protects the cell death induced by various stresses including heat shock and growth factors. SUMO was overexpressed in response to stresses and sumoylation reduced reactive oxygen species (ROS) generation induced by various stresses. This suggests that sumoylation has the opposite function of ubiquitination in response to various stresses even with similar molecular structure. The exact biological function of SUMO should be further studied.

2240

**Oxidative Regulation of Nm23-H1, Tumor Metastasis Suppressor, in Rac1-Induced Reactive Oxygen Species Generation**

A. Kim, S. Kim, K. Lee; Center for Cell Signaling Research, Division of Molecular Life Sciences, Ewha Womans University, Seoul, Republic of Korea

Nucleoside diphosphate kinase (NDPK, Nm23), known as tumor metastasis suppressor, is considered a housekeeping enzyme catalyzing transfer of

terminal phosphate from nucleoside triphosphate (NTP) to nucleoside diphosphate (NDP). Nm23 has been implicated as a multifunctional protein involved in cellular differentiation and development as oncogenesis and tumor metastasis. Recently, Nm23 has been reported to act as guanine nucleotide exchange factor (GEF) of Rac1 activation in reactive oxygen species (ROS) production. However, the regulatory mechanism of Nm23 is poorly understood. In our study, we found that enzymatic activity of Nm23 is damaged by oxidation in cysteine residues and repaired by NADPH-thioredoxin reductase-thioredoxin system. Here we have examined whether oxido-reduction of Nm23 could regulate the Rac1 activation in ROS generation by using Nm23 mutants. The results will demonstrate the regulatory mechanism of Nm23 in various biological processes.

2241

**Overexpression of Ref-1 Suppresses NO Synthesis by Reducing Oxidative Stress in LPS-Stimulated Macrophages RAW2647**

S. Park,[1,2] M. Kim,[1] Y. Kang,[1] Y. Yoo,[2] S. Seo,[3] Y. Park,[1] Y. Park[1]; [1]Microbiology and Immunology, Pusan National University College of Medicine, Busan, Republic of Korea, [2]Anatomy and Cell Biology, Dong-A University College of Medicine, Busan, Republic of Korea, [3]Microbiology, Dong-A University College of Medicine, Busan, Republic of Korea

Redox factor-1 (Ref-1) is a ubiquitously expressed protein with proven roles as a modulator of redox-sensitive transcription, and as an endonuclease in the base excision repair pathway of DNA damaged by oxidative stress. Our previous studies indicate that Ref-1 inhibits nitric oxide (NO) synthesis and apoptosis in LPS-stimulated murine macrophages, but the molecular mechanism of Ref-1 in regulating NO synthesis is not known. We successfully overexpressed Ref-1 protein by introducing adenovirus encoding full length Ref-1 or Ref-1 in DsRed expression vector. Intracellular NO and reactive oxygen species (ROS) were detected by fluorescence of DAF-2 DA and DCF-DA using a confocal laser scanning microscope, respectively. Here we report that forced overexpression of Ref-1 in macrophages suppressed ROS generation by the inhibition of LPS-stimulated NADPH oxidase activity that is the most important system for ROS generation in phagocytes. In parallel with this, Ref-1 also mitigated NF-κB transcriptional activity, NO production with changing NO synthase expression, and apoptosis. Our findings provide a functional connection between inhibition of NO synthesis mediated by NF-κB and decrease of intracellular ROS via the inhibition of NADPH oxidase in the activation of macrophages.

2242

**Type 5 Adenylyl Cyclase Disruption Increases Longevity and Protects Against Stress**

L. Yan, D. E. Vatner, A. Ivessa, J. Liu, J. Thaisz-Warden, J. P. O'Conner, M. Brimacombe, H. Ge, W. Chen, I. Yoshihiro, J. Sadoshima, S. F. Vatner; Cell Biology and Molecular Medicine, UMDNJ-New Jersey Medical School, Newark, NJ

In this study, we attempt to reveal the molecular mechanisms mediating longevity in our novel mouse model of longevity in which type 5 adenylyl cyclase (AC-5) is knocked out (AC-5 KO). Kaplan-Meier survival analysis revealed that the median lifespan was increased by 10 months ($p<0.01$) in AC-5 KO mice (34 months) compared to wild type (WT) mice (24 months). Aging AC-5 KO mice also exhibit maintained bone integrity and resistance to the development of aging cardiomyopathy, e.g. hypertrophy and apoptosis. Using a proteomic-based approach including 2D gel electrophoresis, mass spectrometry, and western analysis, we demonstrated a significant activation of the Raf/MEK/ERK signaling pathway and upregulation of cell protective molecules, including the anti-oxidant Mn-superoxide dismutase (MnSOD). Raf-1, p-MEK, p-ERK, and MnSOD were increased by 3 fold, 50%, 6 fold, and 40% respectively, $p<0.05$. Fibroblasts isolated from AC-5 KO mice, when treated by $H_2O_2$ (50-400 μM) or UV light (25 and 100 mJ/cm$^2$), exhibited enhanced resistance to oxidative stress with increased cell survival and reduced apoptosis. The inhibition of ERK activation with the inhibitor PD98059 reduced oxidative stress tolerance and the level of MnSOD in AC-5 KO mice, suggesting ERK activation mediates resistance to oxidative stress and SOD acts as a downstream enzyme of the ERK signaling pathway. Furthermore, we used yeast as a model to study the effects of ERK activation on longevity. Overexpression of mammalian ERK2 in budding yeast resulted in extended lifespan and resistance to heat shock and oxidative stress, supporting our hypothesis that Raf/MEK/ERK signaling pathway plays an important role in regulating longevity. The current investigation suggests that disruption of AC-5 mediates activation of the Raf/MEK/ERK signaling pathway, which in turn results in protection from oxidative stress and apoptosis, leading to lifespan extension.

2243

**Thrombin-Stimulated Interleukin-8 Activation in Human Macrophages is Mediated by Rho/ROCK/JNK Pathway and the NFκB/AP1 Transcription Regulators**

L. Zheng, M. Martins-Green; Department of Cell Biology and Neuroscience, University of California, Riverside, CA

Wound healing is orchestrated by a variety of cell types with macrophages playing crucial roles in chemokine. Inflammatory chemokines, such as interleukin-8 (IL-8), are known to be induced by local cues at wound site and contribute to chemotaxis, tissue remodeling and angiogenesis. We have investigated the molecular mechanisms of thrombin-induced IL-8 expression in human THP-1 macrophages. Immunoblot analysis and RT-PCR show that thrombin stimulates IL-8 in a rapid and dose-dependent manner and that this stimulation occurs via the PAR1 receptor. Various inhibitors were used to delineate the pathways involved in this stimulation. We find that inhibitors of the c-Jun N-terminal Kinase (JNK) strongly inhibit thrombin-induced IL-8 production. JNK is phosphorylated in response to thrombin treatment confirming its crucial function as a mediator. Using affinity pull-down assays we find that that Rho GTPase is activated by thrombin; Rho kinase(ROCK) inhibitor can inhibit both the IL-8 production and JNK phosphorylation suggesting that Rho/ROCK/JNK are involved sequentially in this activation cascade. We are using retrovirus-based tet-on systems to study the Rho function in more detail. Antisense oligos targeting c-Jun and c-Fos block IL-8 production, suggesting that AP-1 is important, which is further confirmed by EMSA. In addition, the NFκB inhibitor dexamethasone strongly inhibits thrombin-induced IL-8 stimulation and the p65 subunit of NFκB translocates into the nuclei after thrombin stimulation. Further test of NFκB EMSA confirmed its role in IL-8 transcription activation. We are currently using ChromatinIP to confirm the interaction of NFκB and AP-1 with IL-8 promoter *in vivo*. Our study broadens the horizon of inflammatory chemokine regulation and may help develop pharmaceutical methods to manipulate such conditions.

2244

**Two Ubiquitin-conjugating Enzyme Variants Regulate Different Cellular Stress Responses**

P. L. Andersen, H. Zhou, W. Xiao; Microbiology and Immunology, University of Saskatchewan, Saskatoon, SK, Canada

The ubiquitin conjugating (Ubc) enzyme, Ubc13, in concert with the ubiquitin conjugating enzyme variant (Uev) Mms2 is essential for DNA error-

free post-replication repair (PRR) in budding yeast by promoting noncanonical lysine-63 ubiquitin polymerization of PCNA. Mammalian cells contain two Mms2 homologs, Uev1A and Mms2, with greater than 90% amino acid sequence identity to the core region and both are able to interact with Ubc13. Furthermore, both Mms2 and Uev1A are capable of substituting for the yeast Mms2 mutant, suggesting the presence of Ubc13-Uev mediated error-free PRR in mammalian cells. Alternatively, in mammalian cells Ubc13 is reported to interact with a Uev (Uev1A or Mms2) to regulate the NF-kB signal transduction pathway by poly-ubiquitinating NEMO/IKKgamma. Based on the above observations, we hypothesized that the Ubc13 activity to ubiquitylate different substrates is dependent on its physical interaction with different Uevs. Using RNA interference (RNAi), myc-tagged constructs and immunocytochemistry, we demonstrated that suppression of either Ubc13 or Uev1A resulted in loss of NEMO polyubiquitination and NF-kB translocation to the nucleus while suppression of Mms2 has not effect. Alternatively, repression of Ubc13 or Mms2, but not Uev1A, resulted in increased numbers of Rad51 positive nuclei, indicating the increase in spontaneous DNA damage. Additionally, Ubc13 co-localizes with myc-tagged Mms2 in response to DNA damage, while myc-tagged Uev1A was not observed to form nuclear foci. Furthermore, removal of the N-terminal unique region of Uev1A resulted in its co-localization with Ubc13 nuclear foci after DNA damage, reminiscent of the Ubc13-Mms2 nuclear foci. We conclude that Mms2 and Uev1A serve as regulatory subunits of the Ubc13-Uev complex and direct it to two distinct pathways, namely the DNA repair pathway to deal with genotoxic stress and the NF-kB pathway in response to non-genotoxic stresses such as bacterial or viral infection.

2245

**IL-4 Induces the Expression of Suppressor of Cytokine Signalling 3 (SOCS3) Complete Isoform in Human Neutrophils and in DMSO-Differentiated PLB-985**

C. Ratthé, D. Girard; INRS-Institut Armand-Frappier, Pointe-Claire, PQ, Canada

Neutrophils are implicated in the inflammation process. Some cytokines can modulate their functions via the Jak/STAT pathway. It was recently discovered that the Jak/STAT pathway was regulated by suppressor of cytokine signalling (SOCS) that provide a feedback mechanism toward the Jaks and/or cytokine receptor. Among the SOCS, SOCS3 seems to be a major player in the negative regulation of neutrophil functions. The objective of this study was to evaluate the expression and modulation of SOCS3 protein following cytokine stimulations in human neutrophils and differentiated PLB-985 cells (PLB-985D). Neutrophils were obtained from healthy blood donors and PLB-985 cells were differentiated toward the neutrophil phenotype with DMSO. Cells were incubated with G-CSF, GM-CSF, IL-2, IL-4 or IL-6. RT-PCR was used to study SOCS mRNA expression and protein expression was studied by Western Blotting after 1D or 2D gel electrophoresis. The results showed that mRNA expression of SOCS3 in neutrophils was increased after 1h of stimulation with G-CSF, GM-CSF and IL-4. IL-6 also increased SOCS3 mRNA in PLB-985D. The mRNA expression was correlated with protein expression in Western Blot experiments. The increased expression of SOCS3 was observed when cells were pre-treated with the MG132 proteasome inhibitor. Cycloheximide reversed the increase expression of SOCS3. Western Blot experiments showed two different bands after 1D-SDS-PAGE, corresponding probably to the two recently identified SOCS3 isoforms. IL-4 increased the complete isoform and this was confirmed by immunoblotting after running 2D-SDS-PAGE. We conclude that SOCS3 mRNA and the complete isoform of the protein have an increased expression in both neutrophil and PLB-985D cells following stimulation with G-CSF, GM-CSF and IL-4. We also suggest that the increased SOCS3 expression depend on *de novo* protein synthesis.

2246

**Interleukin-8-Induced Endothelial Cell Permeability and Transactivation of Vascular Endothelial Growth Factor-2**

M. L. Petreaca, M. Yao, M. Martins-Green; Cell Biology and Neuroscience, University of California, Riverside, Riverside, CA

Permeability of the endothelium occurs early in the angiogenic process due to endothelial cell de-adhesion and contraction, resulting in gap formation. One of the cytokines involved in this process is the angiogenic chemokine Interleukin-8 (IL-8/CXCL8). We found that IL-8 induces permeability in a receptor-dependent manner that results in activation of Vascular Endothelial Growth Factor Receptor-2 (VEGFR2/Flk-1/KDR). This activation is dose-dependent, occurs over time and involves the phosphorylation of VEGFR2 independently of VEGF. These results indicate that IL-8-induced VEGFR2 activation occurs by transactivation of this receptor. To investigate possible mechanisms of transactivation, we investigated whether physical interaction between VEGFR2 and the IL-8 receptors was needed by performing co-immunoprecipitation assays. We found that CXCR2 interacts with VEGFR2 following IL-8 treatment and that the time course of this interaction is comparable to that of VEGFR2 phosphorylation. We are currently determining whether similar interactions occur with CXCR1. We have also found that Src kinases are important in VEGFR2 transactivation, as an inhibitor of these enzymes prevents both IL-8-induced endothelial permeability and VEGFR2 phosphorylation. Therefore, we hypothesized that Src kinases might stimulate transactivation by mediating VEGFR2-CXCR1/2 interactions. However, Src inhibition did not prevent these interactions, suggesting that Src kinases are involved in transactivation, but are not required for the interaction of the two receptor types. We are currently investigating the nature of IL-8-induced, Src-mediated VEGFR2 phosphorylation/activation. In addition, we are elucidating the role of this transactivation in downstream signaling events important in endothelial permeability, such as phosphorylation of adhesion molecules present at the endothelial cell junctions and the activation of molecules involved in endothelial cell proliferation and migration. These findings shed light into the molecular mechanisms of key processes involved in angiogenesis.

2247

**Angiotensin II Induces Tyrosine Phosphorylation of Glucose Regulated Protein-75 in Rat Liver Cells**

J. B. Gunaje, T. Samikkannu, S. Malaroviyam, J. J. Thomas, T. J. Thekkumkara; Pharmaceutical Sciences, Texas Tech University Health Sciences Center at Amarillo, Amarillo, TX

Angiotensin II (AII) is a peptide hormone that exerts a variety of effects on the cardiovascular system. These include regulation of salt and fluid homeostasis, induction of gene expression and promotion of cell growth. Acting through a seven-transmembrane, G-protein coupled receptor, this octapeptide elicits signal transduction pathways, including stimulation of phospholipase C, protein kinase C, Raf-1 kinase, and MAP kinase and components of the JAK-STAT pathway. Since caveolins are know to be involved in receptor mediated signaling by acting as scaffolding proteins, in this study, we initially determined the effect of angiotensin II on caveolin-1 tyrosine phosphorylation in rat liver WB cells. A time course study showed that instead of caveolin-1 protein, the phospho-specific (tyrosine) anti-caveolin-1 antibody cross-reacted distinctly with a 74 kDa protein. We performed studies to establish the identity of this protein. We demonstrate that in Ang II treated cells the 74 kDa protein rapidly undergoes

tyrosine phosphorylation. Its activation by Ang II was detected as early as 5 min, reached maximal at 15 min; however, significantly declined at 30 min. Cell fractionation experiment showed that it is predominantly localized to the cytoplasm. Cytokines/ growth factors (interleukin-6 and epidermal growth factor) failed to induce its tyrosine phosphorylation; however, pervanadate, a stress inducing agent stimulated its tyrosine phosphorylation. Mass spectrometric analysis of the immunoprecipitated protein showed that the 74 kDa protein is glucose regulated protein-75 (GRP75), a member of stress response protein family. Consistent with this data, we observed that GRP-75 undergoes tyrosine phosphorylation in Ang II treated cells. This novel pathway may contribute to Ang II-induced effects in cells expressing Ang II receptors.

2248

**Activation of Signaling Molecules in *Klebsiella pneumoniae*-Infected Human Hepatoma Cells, HepG2**

J. H. Wu, L. Hong, E. Tsai; Microbiology and Immunology, Chang Gung University, Tao Yuan, Taiwan Republic of China

The enterobacterium *Klebsiella pneumoniae* (KP) usually causes urinary tract infection or pneumonia, however in recent years, the bacterium causes severe liver abscess in diabetic patients due to hospital-acquired infection. How this emerging virulent KP strain causes liver abscess is not known. The study investigates signaling pathways in HepG2 cells (as a model cell system) infected by virulent KP. Confluent cell sheets were infected with bacteria suspended in phosphate buffered saline. Cells were harvested at various time intervals and screened for signaling molecules by Western blotting. Inhibitors were also used to examine the pathway. Our results showed that Toll-like receptor (TLR) 2 and 4 are increased 3 min postinfection and the amount continues to increase at 6 h postinfection. Phosphorylated signaling molecules mitogen-activated protein kinase (MAPK) kinase (MEK) 1,2, extracellular- regulated protein kinase (ERK) and p90 ribosomal S6 kinase (p90RSK) were observed and this pathway was inhibited by MEK1,2 inhibitors, U0126 and PD98059. Phosphorylation of another pathway MEK3,6, p38 MAPK and activating transcription factor-2 (ATF-2) was also observed and this pathway was inhibited by p38 MAPK inhibitors, SB203850 and SB202190. The c-Jun NH2-terminal kinase (JNK) pathway and caspase 3 were not activated. Signaling molecule E1K, MAPK- activated protein kinase-2 (MAPKAPK-2) and heat shock protein (HSP) 27 were also not activated. We conclude that the virulent KP infection in HepG2 cells activates signaling pathways through TLR2 and TLR4. Two MAP kinase pathways, the p38 MAPK and ERK pathways, but not the JNK pathway, are involved in the downstream. The infected cells eventually die and the caspase-3 is not involved in the cell death.

2249

**A Conserved Processing Mechanism Regulates the Activity of Transcription Factors Cubitus Interruptus and NF-kB**

L. Tian, A. Matouschek; Department of Biochemistry, Molecular and Cellular Biology, Northwestern University, Evanston, IL

The proteasome degrades most of protein substrates completely into small peptides**.** However, some proteins, such as transcription factors Cubitus interruptus and NF-κB**,** can be degraded partially to generate biologically active protein fragment. Here we have identified and characterized the signals in the substrate proteins that cause processing. The minimum processing signal consists of a sequence stretch with simple amino acid composition preceding a tightly folded domain in the direction of degradation. The strength of the processing signal depends on the complexity of the simple sequence regardless of identity of amino acid, the resistance of the folded domain to unraveling by the proteasome, and an appropriate spacing between the simple sequence and folded domain. We show that two unrelated transcription factors, Cubitus interruptus and NF-κB, utilize this mechanism to undergo partial degradation by the proteasome *in vivo*. These findings suggest that the mechanism is conserved evolutionarily and that processing signals may be widespread in regulatory proteins.

2250

**Lysophosphatidic Acid Inhibits Diarrhea Through CFTR-dependent Protein Interactions**

C. Li,[1] K. Dandridge,[1] A. Di,[2] K. Marrs,[1] E. Harris,[1] J. Jackson,[3] N. Makarova,[1] D. Nelson,[2] G. Tigyi,[1] A. Naren[1]; [1]Physiology, University of Tennessee Health Science Center, Memphis, TN, [2]Neurobiology, Pharmacology and Physiology, The University of Chicago, Chicago, IL, [3]Comparative Medicine, University of Tennessee Health Science Center, Memphis, TN

**OBJECTIVE:** Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel localized at the luminal or apical surfaces of epithelial cells lining the airway, gut, etc., and it is well established that CFTR plays a pivotal role in cholera toxin-induced diarrhea. Lysophosphatidic acid (LPA), a phospholipid present in blood and foods, has been reported to play a vital role in a variety of conditions including gastrointestinal wound repair, inflammatory bowel disease, and diarrhea. The aim of this study was to investigate how LPA-elicited signaling might regulate CFTR function in the gut, and to evaluate the therapeutic utility of LPA in alleviating cholera toxin-induced secretory diarrhea in mice. **METHODS:** A series of cellular and molecular techniques including RT-PCR, immunofluorescence microscopy, pull-down assay, co-immunoprecipitation, surface biotinylation, cAMP measurement, macromolecular complex assembly, peptide delivery, and electrophysiological techniques including iodide efflux assay, short-circuit current measurement, whole cell-attached patch clamping, as well as *in vivo* intestinal fluid secretion measurements in animal models, were used in this study. **RESULTS and CONCLUSIONS:** We show that type 2 LPA receptors ($LPA_2$) are expressed at the apical surfaces of intestinal epithelial cells, where they form a macromolecular complex with $Na^+/H^+$ exchanger regulatory factor-2 (NHERF2) and CFTR through a PDZ-based interaction. LPA inhibited CFTR-dependent iodide efflux through $LPA_2$-mediated $G_i$ pathway, and LPA inhibited CFTR-mediated short-circuit currents in a compartmentalized fashion. CFTR-mediated intestinal fluid secretion induced by cholera toxin in mice was significantly reduced by LPA administration, and disruption of this complex using an $LPA_2$-specific peptide reversed LPA inhibition of CFTR function. Our study is the first one to show that the macromolecular complex (CFTR-NHERF2-$LPA_2$) formed *in vitro* is also physiologically and functionally relevant *in vivo*. Therefore, LPA-rich foods may represent an alternative method of treating certain forms of diarrhea.

2251

**AWP1 Interacts with TRAF2 and Involves in NF-kappaB Activation**

E. Chang, J. Ha, K. Han, Z. Lee, H. Kim; Department of Cellular and Developmental Biology, Institute of Dentistry, College of Dentistry and Brain Korea 21 Program, Seoul National University, Seoul, Republic of Korea

TRAF2 (TNF receptor associated factor 2) transduces signals from members of TNF (tumor necrosis factor) receptor superfamily and is involved in the activation of NF-kappaB, JNK and apoptosis. In yeast two-hybrid screening for TRAF2 binding proteins, we found a novel TRAF2 binding

protein, AWP1, which had been known to be a PRK1 binding protein. AWP1 has the TRAF2-binding consensus sequences at amino acid residues 149-189. FLAG-tagged TRAF2 was co-immunoprecipitated with HA-tagged AWP1 from 293 cell lysate, and in vitro binding assay using the deletion mutants of AWP1 showed that AWP1 directly binds to the TRAF domain of TRAF2. Wild type of AWP1, when overexpressed in MG63 cells, led to NF-κB activation in response to TNF-α and that activation was decreased by dominant negative TRAF2. Together, AWP1 increased IκB-α phosphorylation and degradation but not JNK activation. However, AN1 mutant carrying a deletion of TRAF2 binding domain failed to activate NF-κB, indicating that AWP1 activated NF-κB through binding to TRAF2. More importantely, overexpression of AN1 mutant increased TNF-α-induced apoptosis and decreased the induction of anti-apoptotic molecules including $FLIP_S$ and cIAP-2. These results suggest that AWP1 may be involved in TRAF2-NF-κB signaling pathway, leading to the induction of anti-apoptotic molecules.

2252

**SXXE Motif Phosphorylation within the Death Domain of TNFRI is Critical for TNF-α Signaling**

Y. Guan, Z. Yang, E. Chin; Department of Surgery and Department of Molecular and Cell Biology, Brown Medical School/Rhode Island hospital, Providence, RI

Tumor necrosis factor-α (TNF-α) activated TNF receptor I (TNF-R1, p55) recruits TRADD, which in turn recruits FADD and RIP to form a signaling complex via homologous death domain-death domain interaction. This death domain signaling complex formation is essential for both NF-κB activation and apoptosis induction. To explore whether death domain post-translational modification events are involved in TNF-R1 -TRADD interaction and NF-κB activation, we performed site-directed mutagenesis, protein-protein interaction analysis and *[$^{32}$P]*orthophosphate labeling assay. Our results present here showed that neither of two previously reported phosphotyrosine motifs $Y^{360}$XXV and $Y^{401}$XXL of the TNF-R1 death domain is critical for TNF-R1 in NF-κB activation and apoptosis induction. Instead, we identified an SXXE motif, i.e., $S^{381}$DHE sequence, critical for TNF-R1-TRADD complex formation. TNF-R1 with $S^{381}$->A or $E^{384}$->A substitution, or $S^{381}$XXE motif deletion abolished TNF-R1 -TRADD complex formation and subsequent NF-κB activation or apoptosis induction. The association of TNF-R1 and TRADD was disrupted by a phosphorylated-peptide of TNF-R1 $S^{381}$DHE motif sequence. TNF-R1 phosphorylation on $S^{381}$ site in response to TNF-α treatment was further confirmed in different cell types by using a specific antibody prepared to recognize phospho-$S^{381}$DHE motif of TNF-R1. Moreover, The $S^{215}$ XXD and $S^{296}$XXE motifs of TRADD death domain were responsible for FADD and RIP association, respectively. Therefore, the "SXXD/E" motif-death domain interaction functions as a signaling module during the death domain interaction. The "SXXE" motif phosphorylation within the death domain of TNF-R1 is critical for TNF-α Signaling. .

2253

**Large-Scale Organization of Intracellular Membranes Relevant to Receptor-Mediated Ca2+ Mobilization**

D. Holowka, L. Vasudevan, S. Hammond, B. Baird; Chemistry and Chemical Biology, Cornell University, Ithaca, NY

Immunofluorescence microscopy reveals a novel pool of PIP/PIP2 -containing membranes in RBL mast cells that is proximal to the plasma membrane and contains key proteins that control Ca2+ mobilization, including phospholipase Cg, inositol trisphosphate receptors, SERCA 2, and the a1 subunit of L-type Ca2+ channels. These membranes are typically localized in one or more micron-sized plaques per cell that are sensitive to dispersion by detergents and organic fixatives. Low molecular weight GTPases, including members of the Arf and Rho families, are also localized to these plaques. Several lines of evidence indicate that these plaques participate in IgE receptor-mediated Ca2+ mobilization, including loss of Ca2+ responses in parallel with the loss of these plaques after incubation of cells at 4oC, and the loss of Arf association with these plaques in mutant RBL cells that are defective in Ca2+ mobilization. Similar plaques are detected in a wide variety of cultured mammalian cells and are commonly localized at nodes of axonal-like projections in differentiated N2A neuroblastoma cells. Membranes in these plaques appear to insert into the plasma membrane in response to appropriate stimuli, such as IgE receptor activation.

2254

***Cyclic* Amp and Calcium Interplay as Second Messengers in Melatonin Dependent Regulation of *Plasmodium Falciparum* Cell-cycle**

F. H. Beraldo,[1] F. M. Almeida,[2] A. M. Silva,[2] G. R. S. Celia[3]; [1]Parasitology, São Paulo University, São Paulo, Brazil, [2]Biochemistry, São Paulo University, São Paulo, Brazil, [3]Physiology, São Paulo University, São Paulo, Brazil

The host hormone melatonin increases cytoplasmic $Ca^{2+}$ concentration and synchronizes *Plasmodium* cell cycle (Hotta et al, 2000). Here we show that in *P. falciparum* melatonin induces an increase in cAMP levels and PKA activity (40% and 50% respectively). When red blood cells infected with *P. falciparum* are treated with cAMP analogue 6-Bz- cAMP there is an alteration of the parasite cell cycle. This effect appears to depend on activation of PKA (abolished by the PKA inhibitors 8-BrcAMP-RP isomer, PKI - cell permeable peptide - and H89). An unexpected cross talk was found to exist between the cAMP and the $Ca^{2+}$ dependent signalling pathways. The increases in cAMP by melatonin are inhibited by blocker of phospholipase C U73122, and addition of 6-Bz- cAMP increases cytosolic $Ca^{2+}$ concentration, through PKA activation. These findings suggest that in *Plasmodium* a highly complex interplay exists between the $Ca^{2+}$ and cAMP signalling pathways, but also that the control of the parasite cell-cycle by melatonin requires the activation of both second messenger controlled pathways.

2255

**Mutational Analysis of the Hsp90 co-chaperone Sti1/Hop**

J. L. Johnson, G. Flom, L. Rosen, J. Williams, J. Weekes; Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID

The molecular chaperone Hsp90 plays an essential role in the regulation of numerous signal transduction pathways by promoting the proper folding of interacting client proteins. Through simultaneous interactions with Hsp70 and Hsp90 via separate tetratricopeptide repeat (TPR) domains, the co-chaperone protein Hop/Sti1 has been proposed to play a critical role in the transfer of client proteins from Hsp70 to Hsp90. However, no prior mutational analysis demonstrating a critical in vivo role for the TPR domains of Sti1 has been reported. We used site-directed mutagenesis of the TPR domains combined with a genetic screen to isolate mutations that disrupt the function of Sti1 in Saccharomyces cerevisiae. We found that mutations in the carboxy-terminal DP2 domain or deletion of a conserved residue in TPR2A, previously shown to interact with Hsp90, completely disrupted Sti1 function. Surprisingly, mutations in TPR1, previously shown to interact with Hsp70, were not sufficient to disrupt in vivo functions unless mutations in TPR2B were present within the same protein, suggesting that TPR1 and TPR2B have redundant or overlapping in vivo

functions. Furthermore, overexpression of mutant Sti1 has dramatically different effects on growth of a yeast strain expressing distinct Hsp90 mutant alleles. We are now determining the effect of mutation on interaction of Sti1 with Hsp70 and Hsp90 in vivo and in vitro. These studies will provide critical information about the in vivo importance of the interaction of Sti1 TPR domains with the carboxy-termini of Hsp70 and Hsp90. We will also gain novel insights about the function of the poorly understood TPR2B and DP2 domains of Sti1 and how Sti1 interacts with Hsp90 to regulate its function.

2256

**Genetic Resolution of the Signal Transmission Through the Modular Membrane Receptor**

S. Bibikov; Protein R&D, EMD Biosciences, San Diego, CA

Both eukaryotic and bacterial membrane receptors are comprised of several modules, often parts of the same protein. These modules normally include the sensor, the intermediates, and the response element. The signal is transmitted between the sensor and the response element as a conformational wave, inducing a change in the structure of the protein and resulting in the miniscule movements of its backbone and side chains. Bacterial receptors have a long alpha helical stretch connecting the membrane-embedded part of the protein with the response part, which is used for activation of the histidine kinase CheA. The helices form the dimers, which in turn form the trimers of dimers. Using the E.coli Aer protein as a model we genetically traced the importance of some residues for the signal translocation inside the protein. We developed a plate assay, which allowed fast collection of mutants with various defects in the Aer function. By testing these defects in two different backgrounds we distinguished the mutations, which can be corrected by altering the background rotational pattern of bacterial flagella, and mutations where the defects to Aer transmission were not repairable. Genetic studies associated with the high-throughput mutagenesis might be useful for studies of the signal transmission in large receptors.

2257

**Penetrating-Antibody Functions *in vivo***

Q. Qian,[1] D. Zhang,[2] H. Li,[2] Y. Ke,[2] W. Zhu,[2] B. Jasti,[1] X. Li[1]; [1]Pharmaceutics and Medicinal Chemistry, University of the Pacific, Stockton, CA, [2]Epitomics. Inc, Burlingame, CA

To deliver functional antibody into cells. Purified rabbit monoclonal antibody against procaspase3 (ECA61) was concentrated to 5mg/ml for conjugation. TAT peptide was chemically conjugated to ECA61 using Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC). The TAT conjugated ECA61 (TAT-ECA61) was incubated with U937 cell line for 2 hours. The extent of intracellular delivery was evaluated by Cell-ELISA: the cells after incubation were washed by PBS, and then fixed by 4% para-formaldehyde, and permealized by 0.1% triton-X100. Subsequently, IHC blocking buffer and anti-rabbit 2$^{nd}$ antibody were applied. DAB Solution was used to visualize the delivered antibody inside cells. Intact ECA61 and TAT-ECA61 without permealization treatments were used as controls in the studies. To verify the function of delivered TAT-ECA61, after incubation with TAT-ECA61, U937 cells were treated with Etoposide at 50μg/ml for 3.5 hours to induce apoptosis. The treated cells were measured for caspase3 activity according to the instruction of Caspase3 Cellular Activity Assay Kit. Rabbit IgG conjugated with TAT was studied as control. From the cell morphology, the TAT-ECA61 had no cytotoxicity on cells, even with high concentration and long incubation time. Three groups of cells were used to demonstrate the antibodies were delivered inside cells. The first group incubated with TAT-ECA61 and followed by permealization showed very strong staining with anti-rabbit antibody. In contrast, the second group incubated with TAT-ECA61 but without permealization treatment and the third group incubated with unconjugated ECA61 didn't show any staining. This result indicated TAT-ECA61 was delivered intracellularly, but not on cell surface. The activation of caspase3 of U937 incubated with TAT-ECA61 was affected, compared with control group incubated with control rabbit IgG-TAT conjugate. The TAT peptide can facilitate efficient delivery of rabbit antibody into cells without influence on cell viability, and TAT-ECA61 can keep its function after delivery process.

2258

**Computational Analysis of Transient Signals Governing Cell Behavior**

B. B. Aldridge,[1] G. Haller,[2] P. K. Sorger,[3] D. A. Lauffenburger[1]; [1]Biological Engineering, MIT, Cambridge, MA, [2]Mechanical Engineering, MIT, Cambridge, MA, [3]Biology, MIT, Cambridge, MA

Computational models of signaling pathways are tools that help us explore complex signaling networks quantitatively. One method to decipher entangled signals is to characterize the signals that influence cell fate. For differential equation models, several common methods exist to analyze system behavior in terms of steady-state signals. However, signal transduction networks influence cell decisions via transient signals that dissipate and return to an inactive steady state. Here, we describe a computational method derived from dynamical systems and fluid mechanics with which to analyze quantitatively transient signals and relate them to cell fate changes. As an example, we applied our method to part of the apoptosis-versus-survival decision network, which is controlled by transient caspase signals. Our analysis method allows us to define a separatrix, which separates the signaling-space into regions destined for cell survival or death where steady state analysis was inadequate.

2259

**Calcium Signaling Protein Complexes in Oligodendrocyte Precursor (OP Cell) Membranes Isolated from Rat Cortices**

S. H. Weerth, J. Hauser, J. T. Russell; SCBST, NICHD, NIH, Bethesda, MD

Calcium imaging studies indicated the existence of specialized calcium release sites in OP cells. At these microdomains calcium waves are generated as puffs or sparks. To further investigate the complex machinery underlying calcium signaling events, we analyzed calcium signaling microdomains in membranes of cultured primary glial cells (OP cells and astrocytes). Caveolin-enriched microdomains were isolated from crude membranes by solubilization in 1% Triton-X100 followed by separation on an isotonic Optiprep™ density gradient. Co-localization of calcium signaling proteins located in the plasma membrane, M1, P2Y1, TRPC1, as well as ER receptors IP3R2 and RyR and the scaffolding proteins Homer and Gq with caveolin-1 was demonstrated by analyzing gradient fractions on SDS-PAGE and western blots. We therefore hypothesized, that calcium signaling proteins are organized in signaling complexes connecting microdomains at the plasma membrane and ER. Immuno-precipitaton studies of astrocyte lysates revealed, that the plasma membrane ion channel TRPC1 co-immunoprecipitated together with the ER calcium channel IP3R2 and the scaffolding protein Homer as a complex. In order to verify the existence of such calcium signaling protein

complexes in intact brain, we immuno-purified OP cell membranes from homogenates of rat cortices with an antibody against NG2, a cell surface marker of OP cells. Co-immunoprecipitation studies of solubilized membranes confirmed the interaction of IP3R2, Homer and TRPC1 in these membranes isolated from brain. Future experiments will test the existence of additional calcium signaling proteins in the signaling complex by immunoprecipitation and western blots or analysis of the immunoprecipitate by Mass spectrometry.

2260

**Dynamic Ensembles of Macromolecules Organized in Nanostructures (DEMONS): A New Paradigm for Intracellular Regulation**

I. I. Moraru,[1] G. Huber,[1] J. H. Carson,[2] A. E. Cowan,[2] C. W. Wolgemuth,[1] L. M. Loew[1]; [1]Cell Biology, University Connecticut School of Medicine, Farmington, CT, [2]Molecular, Microbial, and Structural Biology, University Connecticut School of Medicine, Farmington, CT

Many cellular functions depend on the activities of complex multi-molecular structures that can be of two fundamentally different types: (1) highly ordered stoichiometric nanomachines with defined molecular structures, which are typically effectors of specific functions; examples are ribosomes, proteosomes, nuclear pores, flagella, and molecular motors. (2) dynamic ensembles of macromolecules organized into nanostructures (DEMONS), with variable size, composition, structure, and turnover of individual components, which are typically regulators of specific functions; examples are RNA granules, nuclear bodies, signaling platforms, lipid rafts, and MPF aggregates. We hypothesize that DEMONS have common underlying design principles and represent a novel paradigm for flexible regulation of cellular processes. We developed models that predicted the experimentally observed size distribution of DEMONS by using two alternative physical principles. The first is free energy minimization of opposing forces (short range attractions and long range repulsion) similar to recent approaches in the physics of cluster phases. The second is mass action kinetics of multiple ($\geq$ 3) low affinity binding sites with combinatorial connectivity adjusted for steric screening. Both models reveal similarities in critical parameters for assembly of DEMONS of disparate functions, such as MPF aggregates, which regulate cell cycle control, and RNA granules, which are intermediates in RNA trafficking. Specifically, the data highlight that the assembly/disassembly equilibrium is regulated by the relative size and different binding properties between the aggregate's core and its surface, as well as the requirement for multiple types and/or sites of low affinity interactions. Further refinement of these models could be used to suggest strategies for experimental manipulations influencing assembly and functions of DEMONS. Supported by NIH PN1-EY016530.

2261

**Aggregation of Mast Cell Specific Derivatives of the Ganglioside GD1b Alters Lyn and Syk Distribution and Inhibits Activation via FcepsilonRI**

R. O. Castro, F. Matioli, A. M. M. Souza, M. C. Jamur, C. Oliver; Molecular and Cell Biology, FMRP-USP, Ribeirão Preto, Brazil

Lyn is known to be associated with the mast cell specific derivatives of the ganglioside GD1b that are recognized by mAb AA4. Upon cross-linking, FcepsilonRI rapidly translocates into lipid rafts, where it is phosphorylated by Lyn. The present work investigates the relationship between Lyn and Syk and the derivatives of the ganglioside GD1b during mast cell activation via FcepisilonRI. Unstimulated RBL-2H3 cells are spindle shaped and their surface is covered with microvilli. By immunofluorescence Lyn and the derivates of the gangliosides $GD_{1b}$ are homogeneously distributed along the plasma membrane, but rarely colocalize. Fourty five minutes after stimulation, the cells are spread on the substratum and their surface is covered with membrane ruffles. Lyn is now colocalized in the ruffles with the GD1b ganglioside derivatives. A similar analysis was made for Syk, the second protein kinase activated during signal transduction. In unstimulated cells Syk is localized in the cytoplasm. Forty five minutes after stimulation, Syk is recruited to lipid rafts colocalizing with the GD1b ganglioside derivatives in the ruffles. When mAb AA4 is added to cultures of RBL-2H3 cells, at 24h the gangliosides are aggregated into a single cap on the cell surface. Under these conditions, in unstimulated cells there is no colocalization between Lyn or Syk with the capped gangliosides. Forty five minutes after stimulation, little Lyn or Syk is found in the rufles indicating no colocalization with the capped gangliosides. At 24h in the presence of mAb AA4 beta-hexosaminidase release was reduced by 70 % in RBL-2H3 cells stimulated via FcepsilonRI. These data indicate that the aggregation of the derivatives of the ganglioside GD1b alters Lyn and Syk distribution and inhibits mast cell activation via FcepsilonRI.

2262

**Glycogen Particle Formation in the Yeast *Saccharomyces cerevisiae***

K. Tatchell,[1] J. Salamon-Kozubowska,[1] K. S. Thorn,[2] T. Williams-Hart[3]; [1]Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA, [2]Bauer Center for Genomics Research, Harvard University, Cambridge, MA, [3]Biology, Louisiana State University- Shreveport, Shreveport, LA

Glycogen targeting subunits of Type 1 Protein phosphatase (PP1) regulate glycogen metabolism in eukaryotes by tethering PP1 to enzymatic components of the pathway. In mammalian cell types that accumulate high levels of glycogen, glycogen and its enzymatic machinery reside in cytoplasmic glycogen particles. The physiological role of glycogen targeting subunits in glycogen particle formation is not fully understood. The yeast *S. cerevisiae* has four proteins related to mammalian glycogen targeting subunits. Genetic and biochemical studies on one of these, Gac1, have shown that its ability to bind to both PP1 (Glc7 in yeast) and glycogen synthase is necessary for its ability to promote the normal accumulation of glycogen that occurs as cells approach stationary phase. We show here that at least three of these proteins, Gac1, Pig2, and Gip2, have partially redundant roles in regulating glycogen synthesis. Mutant yeast strains deficient in all three proteins fail to accumulate glycogen. Induced expression of a functional GST-Pig2 fusion protein promotes the concomitant accumulation of glycogen and cytoplasmic spots or particles, in which glycogen synthase-GFP and phosphorylase-GFP fusion proteins co-localize with a Glc7-Red Fluorescent Protein fusion protein. Glycogen synthase is necessary for particle formation. The particles are stable in the cytoplasm and do not undergo the saltatory movement common to many vesicle compartments. Rapid recovery of Glc7-GFP fluorescence after photobleaching suggests that the particles are not in an irreversible aggregation. Deletion mutations in GST-Pig2 that are predicted to prevent the binding of Glc7 and glycogen synthase fail to induce glycogen synthesis and prevent or alter particle formation, as does a mutation in Glc7 that fails to associate with Pig2. Together, these results indicate that yeast can be used to investigate the genetics and cell biology of glycogen particle formation.

2263

### Regulated Cell to Cell Variation During the Response of *S cerevisiae* to Mating Pheromone

A. A. Colman-Lerner,[1] A. Gordon,[1] E. Serra,[1] T. Chin,[1] O. Resnekov,[1] D. Endy,[2] C. Pesce,[1] R. Brent[1]; [1]Molecular Sciences Institute, Berkeley, CA, [2]Division of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA

We studied the quantitative behavior and cell-to-cell variability of a prototypical eukaryotic cell fate decision system, the mating pheromone response pathway in yeast. We dissected and measured the sources of variation in system output analyzing thousands of individual, genetically identical cells. Only a small portion of total cell-to-cell variation was caused by random fluctuations in gene transcription and translation during the response ("expression noise"). Instead, variation was dominated by differences in the capacity of individual cells to transmit signals through the pathway ("pathway capacity") and to express genes into proteins ("expression capacity"). Cells with high expression capacity expressed genes at a higher rate and increased in volume more rapidly. In addition, our results revealed two mechanisms that regulate cell-to-cell variation in pathway capacity. First, the pathway MAP kinase Fus3 suppressed variation at high pheromone while the MAP kinase Kss1 enhanced variation at low doses. Second, pathway and expression capacity were negatively correlated, suggesting a compensatory mechanism by which pheromone-induced gene expression was less influenced by the large variation in expression capacity.

2264

### The Role of Yeh2 in *S cerevisiae* Yeast Mating and Filamentation Signaling Pathways

D. D. Lee, A. D. Palmer, G. G. Hutchins; Biology, Fort Lewis College, Durango, CO

The objective of this study is to characterize the potential role of Yeh2 in the *S. cerevisiae* yeast mating and filamentation signaling pathways. Previous work established Yeh2 as a steryl ester hydrolase in the plasma membrane (JBC 280: 13321-13328). In this study, Yeh2 was found to bind the Ste7 MAP kinase kinase of both mating and filamentation signaling pathways using a yeast 2-hybrid assay. Using a *yeh2* deletion strain, mating efficiency was measured relative to wild type by counting the % cells forming mating projections and undergoing G1 arrest in response to mating pheromone. Localization of Yeh2 protein during formation of mating projections was determined using a fusion of green fluorescent protein (GFP) to Yeh2. Using a cell adherence assay as an index of expression of the flocculation protein Flo11 required for filamentation, the role of Yeh2 in the yeast filamentation response was determined using a *yeh2* deletion either alone or in combination with a *ste5* deletion to minimize cross-talk from the mating pathway. Mating efficiency in a *yeh2* deletion strain was found to be slightly decreased but comparable to wild type. In response to mating pheromone, Yeh2-GFP relocalizes to the tip of the mating projection. In the cell adherence assay , both *yeh2* and *ste5* deletion strains demonstrated increased adherence compared to wild type, whereas a *yeh2 + ste5* deletion strain showed decreased adherence relative to wild type. In summary, Yeh2 appears to be involved in mating pathway signaling given that the *yeh2* deletion strain is modestly decreased in pheromone response relative to wild type and also that Yeh2-GFP localizes to the tip of the mating projection. Yeh2 may also be a regulator of the yeast filamentation response through its involvement in Flo11 expression.

2265

### Expression Profile of Signal Transduction-Related Genes in Phytohormone Treated Sugarcane Plants

F. R. Rocha,[1] F. S. Papini-Terzi1,[1] M. Y. Junior,[1] R. Z. Vêncio,[2] C. S. Rocha,[3] R. Vicentini,[3] V. E. Junior,[4] E. C. Ulian,[5] M. Menossi,[3] G. M. Souza[1]; [1]Instituto de Química - USP, São Paulo, Brazil, [2]Instituto de Matemática e Estatística - USP, São Paulo, Brazil, [3]Centro de Biologia Molecular e Engenharia Genética - Unicamp, Campinas, Brazil, [4]Instituto Agronômico de Campinas, Campinas, Brazil, [5]Centro de Tecnologia Copersucar, Piracicaba, Brazil

The cultivated sweet cane is a hybrid of Saccharum officinarum and Saccharum spontaneum in which a gene can be represented by 10-14 copies. To expedite sugarcane genome research the SUCEST Project sequenced 238 thousand ESTs. Sugarcane's biomass makes it the world's largest crop but despite its economical importance very few studies describe the molecular signaling responses to the environment and the role of phytohormones in this process. The SUCEST-FUN project aims to associate function to sugarcane's genes and to identify putative targets for variety improvement (http://sucest-fun.org). In this work we describe genes responsive to phytohormones that may have a role in the control of metabolic, developmental and defensive processes. cDNA microarrays were used to profile plantlets submitted to methyljasmonate (MeJA) and abscisic acid (ABA) treatments. Sugarcane plantlets were sprayed with a 100 µM MeJA or ABA solution and leaves were collected 0h, 1h, 6h and 12h after treatment. The experiments were conducted with biological replicates and expression profiles clustered by SOM (Self Organizing Maps). Thirty-five differentially expressed genes were selected for their strikingly altered pattern. Among these, we detected 6 genes with no hits in the public databases, 6 stress-related genes and 10 protein kinases (including three receptors). Six of the differentially expressed genes have their transcripts enriched in the leaf tissues. There are three receptors and one protein kinase responsive to the ABA treatment that could not be assigned to known categories based on BLAST searches, but that were assigned to putative families based on a phylogenetic approach. Expression data were validated by real-time PCR. The data presented can be useful in assigning function for the sugarcane genes and reveal potential targets for genetic improvement of sugarcane varieties.

## Cell Cycle Controls II (2266-2291)

2266

### Characterizing the Specificity of the *S cerevisiae* Wee1 Homolog, Swe1p, *in vivo*

M. A. Keaton, D. J. Lew; Pharmacology and Cancer Biology, Duke University, Durham, NC

The execution and proper timing of cell cycle events is due, in part, to the accumulation of specific cyclins that bind and activate cyclin dependent kinases (CDKs). In addition to cyclin binding, CDK complexes are regulated by inhibitors such as the Wee1 family of kinases which phosphorylate a conserved tyrosine residue in the CDK. In most eukaryotes, Wee1 is important for regulating entry into mitosis by inhibiting the mitotic CDK complex. In *Saccharomyces cerevisiae*, overexpression of the Wee1 homologue (Swe1p), arrests cells with duplicated DNA, short spindles, and elongated buds, which is similar to the phenotypes obtained by elimination of the mitotic cyclins Clb1p and Clb2p. This observation combined with studies that have shown that Swe1p preferentially phosphorylates the mitotic Clb2p/Cdc28p complex over the G1 Cln2p/Cdc28p complex *in vitro*, suggests that Swe1 preferentially phosphorylates the mitotic B-type cyclin, Clb1p and Clb2p, complexes. However, the specificity of Swe1p

phosphorylation for the other B-type cyclins has not been evaluated. Using TAP-tag purification and phospho-specific antibodies, we have analyzed the phosphorylation status of a panel of cyclin/Cdc28p complexes *in vivo*. Our results are consistent with preferential phosphorylation of Clb2p complexes over G1 cyclin complexes. We also demonstrate that Clb3p and Clb4p complexes, which are important for spindle formation, are also phosphorylated by Swe1 but to a lesser degree relative to Clb2p complexes. Surprisingly, we found that Clbp5p/Cdc28p complexes have a similar degree of phosphorylation relative to Clb2p complexes when the Cdc28p phosphatase, MIH1, is deleted. This phosphorylation does not appear to inhibit Clb5p/Cdc28p activity since DNA replication is unaffected in *mih1*Δ cells.

2267

**Regulation of Yeast Cbk1 and its Role in Ace2-Driven Transcriptional Asymmetry**

J. Jansen, M. Barry, E. L. Weiss; Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL

The yeast Ndr/LATS family protein kinase Cbk1 is required for sustained polarized growth and daughter cell specific transcription that occurs during mitotic exit. It is part of a novel signaling pathway called the RAM network, which is at least partly conserved from yeast to mammals. Cbk1 promotes asymmetric segregation of the transcription factor Ace2, likely inhibiting its nuclear export in daughter cells; this function requires the Mitotic Exit Network (MEN). We found that blocking nuclear export eliminates Ace2's asymmetry, but does not affect the distribution of Cbk1. Thus, partitioning of the kinase to daughter cells is likely a primary determinant of Ace2 asymmetry. We found that Cbk1 phosphorylates Ace2 *in vitro*, and have identified a possible site of this modification. Ace2 that cannot be phosphorylated at this site is not fully functional *in vivo*. We have also found that two conserved phosphorylation sites in and just C-terminal to Cbk1's kinase domain are important for the kinase's *in vivo* function. Our *in vitro* studies suggest that one site is probably autophosphorylated, while the other is most likely modified by an unknown upstream kinase. Mutation of either site abrogates Cbk1 function; we are investigating localization of these mutant alleles as well as their interaction with other RAM network components. Using antibodies specific for these two phosphorylated motifs, we found that kinase domain site phosphorylation is relatively constant, while C-terminal site modification is more dynamic. The C-terminal site is most dramatically phosphorylated as cells pass from M to G1, and is dephosphorylated in G1. At mitotic arrest, however, C-terminal site phosphorylation is not detectable, indicating that it occurs during mitotic exit. Thus, differential phosphorylation of Cbk1 regulatory sites may be an important way in which this kinase's *in vivo* function is modulated.

2268

**Characterization of a Pathway Required for Septin Organization in Budding Yeast**

K. L. Artiles, D. Kellogg; MCD Biology, UCSC, Santa Cruz, CA

The septins are a conserved family of proteins that are required for cytokinesis and for bud morphogenesis in budding yeast. The septins appear to execute their functions at the bud neck, and an intricate signaling network is required for cell cycle-dependent changes in septin localization to the bud neck. This network includes the protein kinases Elm1, Cla4, Hsl1, and Gin4. The Gin4 kinase binds to the septins and directly phosphorylates the Shs1 septin; however, the functional significance of Shs1 phosphorylation is unclear. In addition, the mechanisms that lead to activation of Gin4 are largely unknown. Genetic and biochemical data suggest that Gin4 is directly activated by the Elm1 kinase, and that Gin4 regulates septin organization by phosphorylating Shs1. We have found that Gin4 is likely to be activated via phosphorylation of an activation loop in the kinase domain, and we are testing whether Elm1 can directly phosphorylate the activation loop. We are also testing whether Gin4 regulates septin organization through phosphorylation of Shs1. However, biochemical and genetic data thus far argue that Gin4 may regulate septin organization via phosphorylation of proteins other than Shs1.

2269

**Characterization of Distinct Sub-populations from *S Cerevisiae* Stationary-Phase Cultures: Identification of a Programmed, Developmental Response to Carbon Starvation**

C. Allen,[1] S. Büttner,[2] O. Mireilles,[1] J. E. Jaetao,[1] M. Veenhuis,[3] F. Madeo,[2] M. Werner-Washburne[1]; [1]Biology Department, University of New Mexico, Albuquerque, NM, [2]Institute of Molecular Biology, Biochemistry, and Microbiology, Karl-Franzens University, Graz, Austria, [3]Department of Eukaryotic Microbiology, University of Groningen, Haren, The Netherlands

The use of *S. cerevisiae* stationary-phase cultures for studies of important processes such as cell cycle, quiescence, aging, and apoptosis has been limited by the heterogeneous mix of cell types in these cultures. In fact, yeast cells were not thought to have a true quiescent ($G_0$) state because of the budded cells in these cultures. The objective of this study was to separate quiescent cells from other cell types in stationary-phase cultures. Two distinct sub-populations of cells were identified in stationary-phase cultures by density-gradient centrifugation. Cells from the heavier fraction were uniform in size, refractile by phase microscopy, mostly unbudded, resistant to cell-wall digestion, and thermotolerant. TEM revealved that the heavier fraction cells had abundant glycogen stores and lacked many internal organelles. Essentially all of these cells were viable and synchronously re-entered the cell cycle. In contrast, the lighter cell fraction contained a mixture of budded and unbudded cells that were sensitive to heat stress, cell wall digestion, lacked glycogen stores, and a majority were senescent. Microarray analysis identified 16 genes that were highly associated with the heavier fraction including several uncharacterized ORFs. Deletions of some of these genes are known to exhibit long-term growth defects, suggesting that these genes may play as yet uncharacterized roles in stationary phase survival. Twelve of thirty genes highly associated with the lighter fraction were involved in TY element transposition, suggesting that these cells are highly recombinogenic, a finding similar to that seen in tumor progression models. The presence of two very distinct cell populations from stationary-phase cultures suggests there is a programmed developmental process during entry into stationary phase leading to production of quiescent daughter cells and non-quiescent, senescent, necrotic, and apoptotic mother cells.

2270

**Microarray Analysis of Gene Activity in *Saccharomyces cerevisiae* Metallo-Aminopeptidase Gene Deletion Strains**

S. F. Roalofs, D. R. Caprioglio; Biology, Colorado State University Pueblo, Pueblo, CO

Metallo-Aminopeptidase gene family 1 (M1AP) encode for peptidases that cleave 1-3 amino acids from the N-terminus of a peptide. M1AP inhibitors have been used in the treatment of cancers and previous data indicate that M1APs may be involved in the cell cycle. Earlier work from

this laboratory showed a G2/M phase delay in strains lacking one or more of the four M1APs (YHR047c (AAP1'), YKL157w(APE2), YIL137c and YNL045w). To investigate the effects of M1APs in the cell cycle, *Saccharomyces cerevisiae* strains were developed with gene mutations in these open reading frames. Strains lacking either ape2 or aap1' were analyzed for mRNA accumulations using microarrays. The wildtype and strains lacking aap1 or ape2 were grown to a log phase in YPD (1% yeast extract, 2% peptone and 2% dextrose) and collected. Total RNA was purified and cDNA probes were made to hybridize to microarrays. Our data showed that mRNA abundances in cell signaling and spindle/bud formation were affected. Random over expressed and under expressed mRNA abundance identified by microarrays were also analyzed by quantitative reverse-transcriptase PCR. These results are consistent with a role of the aminopeptidases in the G2/M phase of the cell cycle. The identification of changes in mRNA abundance in specific genes involved in cell cycle due to M1AP mutations may provide insights into the molecular events leading to the G2/M phase delay. (Supported by NCI Grant P20 CA91489-01A1 and SFR supported by NCI Cancer Education Grant R25 CA49981)

2271

**Cellular Localization of Kic1 Protein Kinase during the Cell Cycle in *Schizosaccharomyces pombe* Fission Yeast**

Z. Tang, A. Tsurumi, B. Ngo, S. Alaei, H. Palomino, C. Barragan; W.M. Keck Science Center, Claremont Colleges, Claremont, CA

Protein kinase Kic1, the orthologue of mammalian CLK/STY1 (Cdc2-like kinase or serine/threonine/tyrosine kinase), regulates the growth polarity, cell surface and septum formation, as well as the late steps of cytokinesis in *S. pombe* cells. We thus hypothesize that the cellular localization of Kic1 is altered at different stages of the cell cycle to play its specific role in cell growth and separation, as a possible mechanism to regulate Kic1 activity during the cell cycle. We examined this mechanism by using GFP tagging approaches through either ectopical expression of the N-terminal GFP-tagged Kic1 or genomic insertion to generate C-terminal GFP-tagged Kic1. A plasmid containing *GFPkic1*$^+$ gene was transformed into a wild type strain and a number of temperature-sensitive *cdc* (cell-division cycle) mutants. GFP-Kic1 fusion protein was induced for expression and visualized under a fluorescence microscope. The majority of GFP-Kic1 is concentrated in the nucleus of asynchronous wild-type *S. pombe* cells. Interestingly, GFP-Kic1 often appears in the nucleus as three small dots, suggesting that the protein may be associated with the chromosomes. GFP-Kic1 remains in the nucleus during the S phase; however, it moves towards the poles of the dividing cells at metaphase. Furthermore, when *cdc10*$^+$ or *cdc25*$^+$ gene is inactivated at the restrictive temperature and the cells are blocked at $G_1$ or $G_2$ phase, respectively, GFP-Kic1 is excluded from the nucleus or spread throughout the cell. The results can be validated by the genomic GFP-tagging approach. Our data provide the evidence that the cellular localization of Kic1 changes at various stages of the cell cycle and it is dependent on the activities of the important cell cycle regulators, Cdc10 and Cdc25, thereby modulating its activity as a kinase to function in cell growth and separation.

2272

**Swe1p Nucleocytoplasmic Shuttling Ensures Updated Communication Between Bud Status and Mitosis**

A. R. Marquitz, D. J. Lew; Duke University, Durham, NC

The *S. cerevisiae* mitotic inhibitor Swe1p accumulates both in the nucleus and at the mother-bud neck. Neck targeting requires Hsl1p, and triggers Swe1p degradation. When bud emergence is impaired, Hsl1p is inactive, Swe1p accumulates in the nucleus and inhibits mitosis until a bud has formed. This coupling of mitosis to bud formation is called the morphogenesis checkpoint. We show that Swe1p shuttles in and out of the nucleus, and identify the NLS and NES sequences responsible. NES mutation rendered Swe1p constitutively nuclear and resistant to Hsl1p-mediated degradation. NLS mutation rendered Swe1p constitutively cytoplasmic and still subject to Hsl1p-mediated degradation. As the Swe1p-targeted cyclin/CDK is primarily nuclear, we suggest that Swe1p shuttles so that it can sense budding status (in the cytoplasm) and convey that information to its target (in the nucleus). NLS mutation rendered Swe1p ineffective in promoting G2 arrest, presumably because of reduced access to its target. Although mutation of the NES should place Swe1p together with its target, we found that this also rendered Swe1p significantly less potent as a mitotic inhibitor. Possibly, Swe1p needs to shuttle in order to access both nuclear and cytoplasmic pools of its target. However, we found that even combined expression of an NLS mutant and an NES mutant of Swe1p in the same cell did not restore Swe1p potency. These findings suggest that Swe1p must shuttle from nucleus to cytoplasm (rather than simply populating both) in order to be fully active. We suggest that nuclear Swe1p is gradually inactivated, and must periodically return to the cytoplasm for reactivation. This constitutes a "freshness dating " mechanism which ensures that only Swe1p that has sampled the budding status recently can promote arrest.

2273

**A Cortical Microtubule Capture Protein and a Kinesin Monitor Spindle Position and Regulate Mitotic Exit in Budding Yeast**

S. Nelson, J. A. Cooper; Cell Biology and Physiology, Washington University, St. Louis, MO

In budding yeast, the spindle position checkpoint prevents mitotic exit until the mitotic spindle enters the mother / bud neck. The integrity of the spindle position checkpoint depends on Bud6. To investigate how Bud6 contributes to the checkpoint, we disrupted all of the several cellular processes in which Bud6 is known to have a role. Microtubule / cortex interactions mediated by Bud6 appear to be important for the checkpoint, while bud site selection and actin cable formation do not. We observed that the kinesin Kip2 is required for Bud6-microtubule interactions, and that Kip2 is required for checkpoint function. Genetic analysis placed Bud6 and Kip2 in the same pathway with regard to the checkpoint. We investigated the mechanism of Bud6 checkpoint function by deleting regulators of Tem1, the GTPase that activates the mitotic exit network. The *bud6* and *kip2* checkpoint defects depend on the putative GEF, Lte1, suggesting a model in which microtubule / cortex interactions mediated by Bud6 and Kip2 inhibit Lte1 to delay mitotic exit until the appropriate time. Other known regulators of Lte1, including the GTPases Ras1 and Ras2 and the PAK kinase Cla4 have checkpoint defects of the same magnitude and in the same genetic pathway with Bud6. In addition, loss of Lte1 suppresses the checkpoint phenotypes of all of these mutants. We propose that these proteins interact in a signaling network upstream of the mitotic exit network to monitor spindle position and regulate Tem1 activity.

2274

**Expression of Cell Cycle Regulators and Esophageal Squamous Cell Carcinoma**

Y. Sato,[1] F. Casarotti,[1] C. F. Nascimento,[1] S. Ferreira,[1] R. A. Sallum,[2] F. Soares[1]; [1]Department of Anatomic Pathology, Medical and Research Center Hospital do Cancer A.C. Camargo, São Paulo, Brazil, [2]Department of Abdominal Surgery, Medical and Research Center Hospital do

Cancer A.C. Camargo, São Paulo, Brazil
Esophageal squamous cell carcinoma (ESCC) is highly prevalent in Brazil and responsible for high mortality index. There are very few studies on cyclin expression and clinicopathological data in ESCC. Tumorigenesis is related to deregulation of the cell cycle and alterations of proteins involved in cell cycle control are found in this tumor, as cyclins. This study investigated immunohistochemical expression of cyclins A, B1 and D1 in ESCC and correlated with clinicopathological findings of 579 cases. Immunohistochemistry were carried out using polyclonal cyclins A, B1 and D1 antibodies. Cases represented by surgical resections were performed in three tissue microarray paraffin blocks spotted in duplicate and cases with small biopsies without surgical resections were performed individually. The cases were scored according frequency and intensity of staining pattern. Our results showed a significant correlation between cyclin B1 expression and lymphatic invasion (p=0,006) and infiltration level (p=0,033). The cyclin B1 negative cases were more frequent in deeper invasion lesions and in cases with lymphatic invasion. Cyclin D1 immunoexpression correlated significantly with ethnia (p=0,026), being more expressed in caucasian patients. Although it was not statistically significant, there was a tendency to correlate histological grade and this marker (p=0,070), which was more expressed in well/moderate than in poorly differentiated cases. No significant correlation was observed between cyclin A expression and clinicopathological data. It were observed significantly correlations between expression of cyclins A (p<0,0001), B1 (p=0,014) and D1 (p<0,0001) and overall survival. Multivariate analyses revealed that only pT (0,030) and gender (p=0,004) are independent prognostic factors. However, when pT was not considered in multivariate analyses, cyclin D1 (p<0,0001), ethnia (p=0,003) and cyclin A (p=0,023) remained as significant prognostic factors. This findings suggests an essential role for cyclins in ESCC prognosis. Funded by FAPESP 04/12360-2 and CEPID 98/14335-2.

2275

### Posttranslational Arginylation Regulates Cell Cycle and Affects Cell Adhesion and Morphology

M. Kozak, A. Kashina; Department of Animal Biology, University of Pennsylvania, Philadelphia, PA
N-terminal arginylation is a poorly understood posttranslational modification that is critical for embryogenesis, cardiovascular development, and angiogenesis in mammals. To understand the physiological role of protein arginylation, we studied the behavior and morphology of arginylation-deficient mouse embryonic fibroblasts in culture. We found that the absence of protein arginylation results in a dramatic increase in cell proliferation rate. While the M-phase in arginylation-deficient cells is slowed by approximately 25% compared to control, more cells are entering division in such cultures at a given time. This effect is independent on cell density; when the dividing cells reach a monolayer, their proliferation rates remain high, resulting in the formation of multilayered cultures. Thus, arginylation deficient cells display defects in contact inhibition and cell cycle control, possibly by affecting cell adhesion properties and cell cycle checkpoint(s). To study possible changes in cell adhesion, we examined the morphology of individual cells and found that the absence of arginylation results in the decreased ability of cells to spread on the substrate and form a lamella, accompanied by changes in the extracellular matrix and focal adhesions. Immunoprecipitations of several cytoskeletal proteins reveal differences in polypeptide composition between wild type and arginylation deficient cells, suggesting that arginylation affects intracellular properties of the major cytoskeletal components. Detailed studies of cell cycle changes and identification or possible cell cycle regulators affected by arginylation are now in progress.

2276

### RNF2, an E3 Ubiquitin Ligase, Interacts with D-Prohibitin and Is Involved in the CP2-Mediated Transcriptional Pathway

S. Lee, S. Kang; Graduate School of Biotechnology, Korea University, Seoul, Republic of Korea
It has been reported that RNF2, an E3 ligase protein, has a role in the transcriptional regulation as well as in the ubiquitination proteasome system. Here, we found that RNF2 interacts with the D-prohibitin protein, an inhibitor of cell proliferation, in the CP2 transcriptional pathway. Further experiments using RNA interference and immunoprecipitation assays showed that the stability of CP-2 protein is increased by expressing RNF2 and that D-prohibitin forms a trimeric complex with CP-2 through binding to RNF2. Our fractionation experiments showed that the RNF2 proteins are distributed in the mitochondria as well as in the nucleus. Under the apoptosis condition, however, the RNF2 proteins in the nucleus were exported into the cytoplasm. Our results suggest a novel mechanism by which RNF2 modulates the D-prohibitin/CP2-mediated transcriptional pathway and RNF2 might have multiple functions depending on its interacting proteins, which could be determined by the location of RNF2, mitochondria or nucleus.

2277

### Quantitative Analysis of a Cell Cycle Checkpoint Response in *Xenopus laevis* Cell-Free Egg Extracts

I. Auckland, A. Dravid, J. J. Tyson, J. C. Sible; Biology, Virginia Tech, Blacksburg, VA
At the midblastula transition or MBT in *Xenopus laevis* embryos, cell cycle checkpoints are acquired. The mechanism responsible for the initiation of this important feature of the cell cycle is still not fully understood, but depends in part upon the concentration of nuclei. To develop a more quantitative understanding of the role of DNA in cell cycle checkpoint engagement, we investigated effects of nuclear concentration upon the underlying kinetics of the cell cycle engine in cell-free egg extracts. We quantified these effects by evaluating the threshold concentrations of cyclin required to enter mitosis and the lag times between addition of cyclin and entry into mitosis. We determined that 1) the concentration of nuclei affects lag time of entry into mitosis, 2) cyclin B thresholds required to enter mitosis increases with increasing concentrations of nuclei, and 3) elevated cyclin thresholds caused by DNA replication blocks are further increased by increasing the concentration of nuclei. In the developing embryo, the nuclei concentration doubles every 30 minutes and could be responsible for the introduction of gap phases and checkpoints at the MBT. This study examines the effect of nuclear concentration in the *Xenopus laevis* cell-free egg extract system with and without unreplicated DNA. Paired with our ongoing development of mathematical models of cell cycle networks, these studies will help build a quantitative, systems-level view of the regulation of cell cycle and its interaction with checkpoint signaling networks.

2278

### Chk1 Controls DNA Damage-induced Centrosome Amplification

E. Bourke,[1] A. Merdes,[2] L. Cuffe,[1] H. Dodson,[1] G. Zachos,[3] D. Gillespie,[3] C. Morrison[1]; [1]Biochemistry, NUIG, Galway, Ireland, [2]CNRS-Pierre Fabre, Toulouse, France, [3]Beatson Institute for Cancer Research, Glasgow, United Kingdom

Numerical aberrations of the centrosome, the key microtubule organising centre of animal cells, are commonly observed in cancers and in cells with defective DNA repair. Here we show by light and electron microscopy that gamma irradiation induces centrosome amplification in human cells. Such amplification can be abrogated by caffeine in both ATM- and ATR-defective cells, suggesting a complementary role for these two DNA damage-sensitive kinases in permitting this response. We demonstrate that treatment with UCN-01, a CHK1 inhibitor, suppresses centrosome amplification after DNA damage in human cells. Radiation-induced centrosome amplification was abrogated in Chk1-/- DT40 cells, but occurred at normal levels in Chk1-/- cells expressing a Chk1 transgene. Expression of kinase-dead Chk1, or Chk1S345A, through which the PI3K kinases cannot signal, failed to restore the capacity for centrosome amplification, suggesting that both PI3K signalling to Chk1 and Chk1 activity are necessary for centrosome amplification to occur after DNA damage. Using cold treatment to arrest the cell cycle without causing DNA damage, we show that a G2 arrest alone is not sufficient to potentiate significant centrosome amplification, suggesting that a Chk1 signal may act directly on the centrosome in allowing amplification after DNA damage. These findings suggest that Chk1 plays a key role in maintaining genomic integrity through controlling the centrosome cycle in response to DNA damage.

2279

**Mammalian SWI/SNF Complexes Enhance DNA Double-Strand Break Repair by Promoting γ-H2AX Induction**

J. Park, E. Park, J. Kwon; Division of Molecular Life Sciences, Ewha Womans University, Seoul, Republic of Korea

Mammalian SWI/SNF complexes can modulate chromatin structure by utilizing the energy of ATP hydrolysis and their role in transcription has been well established. However, the role of SWI/SNF in DNA repair has remained largely unexplored. Here we provide evidence that the SWI/SNF complexes enhance DNA double strand break (DSB) repair by stimulating γ-H2AX induction. Inactivation of the SWI/SNF complexes via expression of dominant negative core subunit BRG-1 results in reduced DSB repair with transcriptional modulation associated with DNA repair largely unaffected. SWI/SNF defect severely compromises both H2AX phosphorylation and γ-H2AX focus formation after DNA damage without affecting expression of ATM, DNA-PK and ATR, or their activation and recruitment to DNA breaks. In addition, immunofluorescence data indicate that the formation of γ-H2AX foci following DNA damage is inhibited specifically in the subnuclear regions in which the dominant negative BRG-1 is expressed. These results suggest that the SWI/SNF complexes directly participate in promoting γ-H2AX to enhance DSB repair, providing a novel link between ATP-dependent chromatin remodeling and DSB repair in mammalian cells.

2280

**Determinants of Cell Fate Following DNA Replication Fork Stress**

R. Rodriguez Gonzalez, M. Meuth; Institute for Cancer Studies, University of Sheffield, Sheffield, United Kingdom

Cells respond to DNA replication stress by triggering cell cycle checkpoints, repair, or death. To understand the role of the DNA damage response pathways in determining whether cells survive replication stress or become committed to death, we examined the effect of loss of these pathways on cellular response to agents that slow or arrest DNA synthesis. We show that replication inhibitors such as excess thymidine, hydroxyurea, and camptothecin are normally poor inducers of apoptosis although they are effective inducers of a permanent p53-p21-dependent G2 arrest and senescence in some tumor cell lines. However these agents become potent inducers of death in S-phase cells upon siRNA mediated depletion of the checkpoint kinase Chk1. Cells defective in other proteins that are activated in S-phase following replication fork stress such as ATM, p53, and Chk2 do not show the same apoptotic response. p21 deficient cells on the other hand produce a more robust apoptotic response upon Chk1 depletion. p21 is normally induced in G2 phase, late after thymidine treatment. In Chk1 depleted cells p21 induction occurs earlier and does not require p53. Thus Chk1 plays a primary role in the protection of cells from death induced by replication fork stress while p21 mediates through its role in regulating entry into S-phase. These findings are of potential importance to cancer therapy as we demonstrate that the efficacy of clinically relevant agents can be enhanced by manipulation of these signalling pathways.

2281

**Mitotic Delay in Response to DNA Damage in Syncytial *Drosophila* Embryos**

S. Kwak, A. Kelkar, S. Takada, W. E. Theurkauf; Program in Molecular Medicine, UMASS Medical School, Worcester, MA

DNA damage-induced mitotic catastrophe is facilitated by defects in the G1 and G2 checkpoints in mammalian cells. To understand the underlying mechanism, we utilize the early *Drosophila* embryo that has a simple S phase-M phase cell cycle and does not delay progression into mitosis in response to DNA damage. However, these embryos have an elaborate M-phase response to DNA damage that includes active disruption of the centrosome and spindle assembly by a Chk2 kinase-dependent pathway (Sibon et al., 2000; Takada et al., 2003). Here we show that embryos also delay mitotic exit in response to DNA damage. Using time-lapse confocal microscopy, we show that DNA damaging agents lead to cell cycle delays with separated sister chromosomes, elongated spindles, and robust anaphase astral microtubule arrays. In wild type embryos, these delays are induced by relatively high levels of agents that cause DNA strand breaks. By contrast, embryos mutant for the Bloom's syndrome homologue or histone H2AV, which are defective in double strand break repair, are hypersensitive to damage and consistently arrest in anaphase in response to very low levels of DNA damaging agents. During damage-induced arrest, levels of mitotic Cyclin B1 and B3 remain high, indicating that damage blocks Cyclin proteolysis on mitotic exit. Mutations in Chk2 completely suppress damage induced mitotic exit arrest, while mutations in Chk1, ATR and p53 have no effect on this apparent anaphase DNA damage checkpoint. We also show that Cdc20/Fzy is modified by DNA damage in Chk2 dependent manner by 2D gel analysis. We therefore conclude that the *Drosophila* Chk2 tumor suppressor homologue is required for a DNA damage dependent mitotic exit checkpoint that functions during early embryogenesis.

2282

**Inhibition of Calcium-independent Phospholipid $A_2$ Induces a p53-dependent Membrane Phospholipid Turnover Checkpoint**

X. H. Zhang, C. Zhao, K. Seleznev, K. Song, Z. A. Ma; Department of Geriatrics, Mt. Sinai Med Ctr, New York, NY

Phosphatidylcholine (PtdCho) is the most abundant phospholipid in mammalian cell membranes. It is essential for cell viability and its levels vary through the cell cycle. The G1 cells maintain a constant membrane phospholipid content but exhibit a high rate of PtdCho biosynthesis and degradation and this high rate of phospholipid turnover is achieved by the opposing actions between CTP:phosphocholine cytidylyltransferase (CCT) and the group VIA $Ca^{2+}$-independent-phospholipase $A_2$ ($iPLA_2$). However, it is unknown whether this G1 phospholipid turnover is directly

coupled to cell-cycle control. We found that disruption of G1 phospholipid turnover with the $iPLA_2$-specific inhibitor bromoenol lactone, with the expression of the dominant-negative domain of $iPLA_2$, or with siRNA against $iPLA_2$ arrested rat insulinoma INS-1 and human colon carcinoma HCT cells in G1 by activation of the tumor suppressor p53 and expression of the cyclin-dependent kinase inhibitor p21. In contrast, HCT $p53^{-/-}$ cells failed to arrest in G1 in response to inhibition of $iPLA_2$ and HCT $p21^{-/-}$ cells underwent massive apoptosis that can be rescued by downregulation of p53 by siRNA against p53. Furthermore, we found that inhibition of $iPLA_2$-mediated PtdCho degradation induced the rapid phosphorylation of serine-15 of p53 without causing DNA damage, and that this phosphorylation is blocked by caffeine, a specific inhibitor of ATM kinase. Our data indicated that the disruption of phospholipid turnover activates the ATM-p53 pathway, which induces the expression of p21 to prevent CDK activation and subsequent G1 arrest. Thus, we propose that the phospholipid turnover in G1 cells is an intrinsic checkpoint essential for S phase entry, and that this checkpoint is monitored through cooperation of $iPLA_2$ with p53.

2283

**Heterogeneity in Expression and Sequence of SIRT2, p53, and Stat3 Genes in a Spontaneous Canine Model of Breast Cancer**

R. Bird, P. DeInnocentes, L. Ring, L. Li, R. Sanchez; Pathobiology, Auburn University, Auburn, AL

Spontaneous canine mammary cancer (CMT) is the most common malignancy of dogs and a highly homologous model of human breast cancer. We have investigated potential links between genes associated with lifespan, survival and neoplastic potential to better explain changes and loss of proliferative control and expression of the differentiated phenotype associated with neoplastic transformation. Differences in cell lines derived from individual malignant canine mammary tumors were investigated to identify new regulatory genes associated with neoplasia, cell proliferation, apoptosis and senescence. The silent information regulator 2 (SIR2) gene family has been implicated in gene silencing, chromosomal stability, aging and histone deacetylation. SIR2 encodes a multigene family of highly conserved sequences encoding nicotinamide adenine dinucleotide-dependent histone deacetylases. Stat3 encodes a signal-transducer and activator of transcription that is frequently overexpressed in human breast cancer potentiating angiogenesis, regulating cell cycle progression and suppressing apoptosis. Canine SIRT2 rt-PCR revealed abundant transcripts in all cell lines, were most homologous to human SIRT2 and contained non-conserved amino acid substitutions representing mutations or allelic differences and interspecies differences including substitutions of conserved cystein residues in the Zn2+-binding motif. Defects in gene products interacting with SIRT2 were also noted as was an extended G2/M phase. Abundant but variable levels of highly conserved Stat3 were also expressed in these CMT cell lines. Mutations in SIRT2 were coincident with abnormal expression/truncation of p53 its modulator Wip1 and a failure to activate p21/Cip1. This data suggests mechanisms by which regulation of expression could integrate developmental/apoptotic pathways with SIRT2-dependent lifespan and cell proliferation regulation that could promote cell proliferation and reinforce neoplastic transformation. These genes offer promising therapeutic targets that may contribute to transformed and immortalized phenotypes in this spontaneous model of breast cancer.

2284

**Distinct Mechanisms of Cdk Hyperactivity Regulate Invasion and Resistance to Differentiation in Human Neuroblastoma Cell Lines**

E. O'Reilly, F. Barrett, E. Carroll, H. C. Gallagher; Pathology, University College Dublin, Dublin, Ireland

Resistance to differentiation is a key feature of advanced, highly invasive neuroblastoma tumours. This relies on the maintenance of cell cycling, but how the cell cycle machinery controls the critical balance between neuroblastoma proliferation and differentiation is unclear. Here, we dissected the relationship between cyclin signaling and the proliferation, differentiation and invasiveness of four human neuroblastoma cell lines (SH-SY-5Y, SK-N-DZ, SK-N-MC and SK-N-SH). Four cyclin-dependent kinase (cdk) inhibitors (roscovitine, olomoucine, indirubin-3-monoxime and a cdk 4-selective inhibitor) and two pro-differentiative retinoids (all-trans-retinoic acid and 13-cis-retinoic acid) were used. Cell viability/proliferation was assessed by MTT assay and differentiation by neurite outgrowth assay. Invasion assays employed a matrigel matrix. Key cyclins, cdks and inhibitory proteins as well as the profilerative marker, PCNA, and neuronal differentiation markers were analyzed by immunoblotting. We found a strong correlation between hyperactivity of certain cdks and invasiveness in the four cell lines, both basally and in response to drug treatment. In addition, sensitivity to differentiation was drug- and cell line-specific and involved the inhibition of key cdks and their cyclin partners. For example, in SH-SY-5Y cells, cdk4 was over expressed basally and cell cycle exit and differentiation was induced time-dependently by the cdk4 inhibitor and 13-cis-RA, at low micromolar concentrations. However, these cells were more resistant to ATRA, olomoucine, roscovitine and indirubin-3-monoxime (cdk2/5-specific inhibition). SK-N-DZ cells were more sensitive to ATRA than to 13-cis-RA and their differential sensitivity to cdk inhibitors reflected their basal cyclin/cdk expression pattern. Across all cell lines, however, a shut-down in cyclin signaling always accompanied cell differentiation and reduced invasiveness. We suggest that specific mechanisms of cdk hyperactivity in individual neuroblastoma tumours may determine their sensitivity to chemotherapeutic drugs. Moreover, cdk inhibitors may be useful for treating neuroblastoma.

2285

**Cytosolic Phospholipase $A_2$-α Mediates Endothelial Cell Proliferation and is Inactivated by Association with the Golgi apparatus**

S. P. Herbert, S. Ponnambalam, J. H. Walker; Biochemistry and Microbiology, University of Leeds, Leeds, United Kingdom

The formation of new blood vessels from pre-existing blood vessels (angiogenesis) is a multi step process involving endothelial cell (EC) proliferation, migration and differentiation. Arachidonic acid and its metabolites may play a role in a number of these steps. Receptor-stimulated arachidonic acid release is mediated by cytosolic phospholipase $A_2$ ($cPLA_2$), a $Ca^{2+}$-sensitive member of the phospholipase-$A_2$ superfamily of lipolytic enzymes. We tested the hypothesis that $cPLA_2$ activity is linked to the control of EC proliferation. We discovered that $cPLA_2$ plays a central role in the control of EC proliferation (Herbert, S.P. *et al*. (2005) *Mol. Biol. Cell.* Jun-1 [Epub ahead of print]). The specific $cPLA_2$a inhibitor, pyrrolidine-1, inhibits EC proliferation in a dose dependent manner which is reversed upon the addition of exogenous arachidonic acid. Furthermore, only upon proliferation do ECs exhibit any $cPLA_2$ activity. In confluent, non-proliferating ECs $cPLA_2$ is inactivated by a novel mechanism involving association with the Golgi apparatus. Sequestration at the Golgi impedes the association of $cPLA_2$ with its phospholipid substrate at the endoplasmic reticulum. The inability to access substrate effectively inhibits $cPLA_2$ activity by 87% and prevents the functional coupling of $cPLA_2$ with cyclooxygenase enzymes, reducing $PGE_2$ release by 96%. In addition, $cPLA_2$ activity is essential for the re-entry of non-proliferating ECs into the cell cycle. Proliferation will only occur upon activation of $cPLA_2$ by release from the Golgi. We find that $cPLA_2$ activity

then mediates the expression of Ki67, an essential cell cycle regulatory protein. In conclusion, we find that $cPLA_2$ plays a central role in the induction of EC proliferation, a vital aspect of the angiogenic response. Furthermore, $cPLA_2$ activity and thus its ability to induce EC proliferation, is regulated by reversible association with the Golgi apparatus.

2286

### Mitochondrial ATP Synthase Inhibition by 3,3'-diindolylmethane (DIM ) Contributed to the Reactive Oxygen Species (ROS) Production, p38 and JNK Activation and Subsequent $p21^{WAF1/CIP1}$ Upregulation in Human Breast Cancer MCF-7 Cells

Y. Gong,[1] H. Sohn,[1] L. Xue,[2] G. Firestone,[2] L. Bjeldanes[1]; [1]Nutritional Sciences and Toxicology, UC Berkeley, Berkeley, CA, [2]Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Epidemiological evidence suggested that high dietary intake of fruit and vegetables protects against tumorigenesis in multiple organs. Among these vegetables with anticancer properties, those of *Brassica* genus, such as broccoli, cabbage and Brussels sprouts, appear to be very effective in reducing the risks of several types of cancers, including breast cancer. 3,3'-Diindolylmethane (DIM), one of the most abundant and biologically active *in vivo* products derived from *Brassica* vegetables, is a promising antitumor agent. Previous studies in our lab identified $p21^{WAF1/CIP1}$ overexpression as the major trigger of DIM-induced G1 arrest in breast cancer MCF-7 cells. In this study, the upstream events leading to $p21^{WAF1/CIP1}$ overexpression were further investigated. Using pathway specific inhibitors SB203580 and SP600125 and dominant negative expression vectors, we identified stress-activated MAP kinase pathway p38 and JNK as the major upstream pathways leading to DIM-induced $p21^{WAF1/CIP1}$ mRNA transcription. In addition, we demonstrated for the first time that DIM is a strong mitochondrial ATP synthase inhibitor ($IC_{50}$~25μM). DIM treatment induced hyperpolarization of mitochondrial membrane potential and decreased ATP production. Like many other oxidative phosphorylation inhibitors, DIM treatment significantly stimulated mitochondrial ROS production, which led to the activation of redox sensitive p38 and JNK pathway. In further support of the results, when DIM was coadministered with antioxidants vitamin E and C, the activation of p38 and JNK and the overexpression of $p21^{WAF1/CIP1}$ were all attenuated. Our continuing studies seek confirm the role of mitochondrial in DIM-mediated release of ROS and expression of $p21^{WAF1/CIP1}$. The present studies are the first to link mitochondrial ROS release with DIM-induced cell cycle arrest.

2287

### ATP-dependent Proteases Differ Substantially in their Ability to Unfold Globular Proteins

N. E. Jaffe, P. Koodathingal, A. Matouschek; Dept. of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL

Degradation of proteins by ATP-dependent proteases plays a critical role in several cellular processes, including the cell cycle, gene expression, signal transduction and antigen presentation. Proteins must be unfolded to be degraded by ATP-dependent proteases. My research focuses on the mechanism of protein unfolding and destruction by the proteasome and its functional bacterial analogues, ClpAP, ClpXP, Lon, HslUV and FtsH. Each of these proteases is active as a cylindrical structure composed of one or more oligomeric rings, arranged such that the active sites of proteolysis are sequestered. In addition, all ATP-dependent proteases generally function the same way; degradation of a substrate protein occurs in a stepwise process where the protein is first targeted and bound to one end of the protease. The protein is subsequently unfolded and its polypeptide chain is translocated to the active sites of proteolysis. Despite these similarities, we have found that ATP-dependent proteases differ substantially in their ability to unfold proteins. Our research demonstrates that ATP-dependent proteases display a hierarchical range of strengths for unfolding proteins from either end of their polypeptide chain. A protein's overall lifetime in the cell, or its susceptibility to degradation, is conferred by its intrinsic stability, its structure, and a targeting signal. Our data suggest that differences in protease unfolding strength may also contribute to a protein's lifetime. By regulating the concentration of strong or weak proteases, the cell could achieve an additional level of selectivity in protein destruction.

2288

### Biochemical Characterization of the Interaction Between CPAP and 14-3-3 Protein

C. Chen,[1,2] M. Olayioye,[3] G. J. Lindeman,[3] T. K. Tang[2]; [1]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan Republic of China, [2]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan Republic of China, [3]The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria, Australia

CPAP, a centrosomal protein 4.1-associated protein, is a multifunctional protein, originally identified by a yeast two-hybrid screening using protein 4.1 as bait (Mol. Cell. Biol. 20, 7813-7825, 2000). CPAP carries a novel microtubule-destabilizing motif that not only recognizes the plus end of a microtubule but also inhibits microtubule nucleation from the centrosome, suggesting that CPAP may play a role in the regulation of microtubule assembly. Interestingly, CPAP was also reported to interact with STAT5 and RelA, suggesting that CPAP may act as a transcription co-activator. To further examine the putative functions of CPAP, we searched for conserved sequence motifs in CPAP and identified two classical 14-3-3 binding motifs. The 14-3-3 proteins are a family of abundant 28-33-kDa acidic polypeptides that bind and regulate a number of key proteins involved in diverse signal transduction pathways. The interaction of CPAP and 14-3-3 was first confirmed by a yeast two-hybrid system followed by a co-immunoprecipitation experiment. Further analyses revealed that the 14-3-3 binding domain is located at the C-terminal domain of CPAP and Ser-1109 is required for 14-3-3 binding. Mutation of serine 1109 (Ser-1109) to alanine significantly inhibits CPAP binding to 14-3-3. Interestingly, the amount of CPAP/14-3-3 complex was significant reduced in cells arrested at M phase. Furthermore, we observed that Ser-1109 of CPAP can be phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) *in vitro*. Together, these results suggested that the formation of CPAP and 14-3-3 complex is in a cell cycle-dependent manner and possibly through the activation of CaMKII.

2289

### Peptide-based Nano-structure for *in vivo* Delivery of Therapeutic SiRNA Targeting the Cell Cycle Regulatory Protein Cyclin B1

E. Gros,[1] F. Simeoni,[1] M. C. Morris,[1] G. Aldrian-Herrada,[1] Q. N. Nguyen,[2] G. K. McMaster,[2] G. Divita[1]; [1]Molecular Biophysics & Therapeutics, CRBM, Montpellier, France, [2]Genospectra Inc., Fremont, CA

The dramatic acceleration in the identification of new nucleic acid-based therapeutic molecules has provided new perspectives in pharmaceutical research. However, development of these molecules is limited by poor cellular uptake and cellular trafficking or the lack of biological activity of

the compound delivered. With the aim of addressing these issues, we have designed a family of short amphipathic peptides (MPG), which consist of a hydrophobic domain and a hydrophilic domain derived from the NLS of SV40 T-antigen. These carriers when complexed with the siRNA form a stable and reversible "nano-cage" around siRNA through non-covalent interactions, thereby increasing their stability. MPG efficiently delivers siRNA into a wide variety of mammalian cell lines through a process involving membrane disorganization, independently of the endosomal pathway. MPG-carrier is a powerful tool for delivery of siRNA, enabling rapid release of the siRNA into the cytoplasm and promoting robust downregulation of target mRNA. MPG-carriers were applied to the delivery of siRNA targeting the cell cycle regulatory protein Cyclin B1 into cancer cell lines and into a mouse model. When associated with MPG carrier, sub-nanomolar concentrations of siRNA Cyclin B1 significantly knocked down Cyclin B1 protein levels and blocked cancer cell proliferation. Temporal regulation of the siRNA Cyclin B1 was validated by using a novel light-activated, controllable siRNA. Kinetic studies have revealed that siRNA mediated knockdown of Cyclin B1 mRNA results in a cell cycle arrest in early G2. MPG/Cyclin B1 siRNA formulations were shown to block tumor growth in vivo upon intratumoral or intravenous injection. Given the biological properties of these novel vectors , we believe that MPG-based technologies will contribute significantly to the development of fundamental and therapeutic applications.

2290

**Development of a Set of Genetically-Encoded Fluorescent Cell Cycle Biosensors**

A. T. Hahn,[1] J. T. Jones,[2] C. R. Gustavsson,[2] T. Meyer[2]; [1]Molecular and Cellular Phys, Stanford University, Stanford, CA, [2]Molecular Pharmacology, Stanford University, Stanford, CA

In order to monitor the timing of all four phases of the cell cycle in live, unsynchronized cell populations, we developed sensors for G1 and S phases to complement the mitosis biosensor that we designed previously (Jones et al, Nat Biotechnol, 2004). The G1 biosensor consists of the carboxy terminus of human DNA helicase B (Gu et al, Mol Biol Cell, 2004) attached to a fluorescent protein. The S phase biosensor is a modified version of the proliferating cell nuclear antigen construct originally developed by Leonhardt and colleagues to monitor the dynamics of replication foci (J Cell Biol, 2000). Using an epifluorescence microscope equipped with a live cell chamber, we have followed unsynchronized cells for the duration of an entire cell cycle, monitoring the spatio-temporal dynamics of the sensors. In G1, the G1 and S phase biosensors are localized to the nucleus. As the cell enters S phase, the G1 biosensor translocates into the cytoplasm where it remains until M phase, while the S phase biosensor forms nuclear puncta that change location and size during S phase progression. In G2, the large puncta from late S phase disappear and the sensor is again localized homogenously in the nucleus. After nuclear envelope breakdown at the start of mitosis, the G1 and S phase biosensors are localized throughout the cell. Using the visual cues provided by these biosensors, we were able to successfully identify and time the durations of all four phases of the cell cycle, a feat previously impossible using standard methods like FACS and antibody staining. This set of biosensors, together with the tracking and analysis software that we have also developed, enable the high content screening of potential anticancer therapeutics and small interfering RNA for proteins involved in tumorigenesis.

2291

**Down-Regulation of the Tumor Suppressor p53 in HEK Cells by Gravity Mechano-Stimulation**

J. Phillips, N. Fingland, E. A. C. Almeida; Life Sciences Division, NASA Ames Research Center, Moffett Field, CA

The tumor suppressor p53 accumulates in tissues exposed to microgravity (Ohnishi et al. 2000). Similar results are seen in studies with hindlimb-unloaded animals, a ground-based model of microgravity (Siu and Alway, 2005). Previous work also shows that hindlimb-unloaded p53-null animals do not exhibit bone loss normally associated with disuse (Sakai et al. 2002). These studies suggest a role for p53 in regulating bone tissue, where the accumulation of p53 negatively regulates bone mass. Since the removal of mechanical forces due to gravity results in the accumulation of p53, we postulate that increased mechanical load from an enhanced gravitational field will deplete p53 levels. To test this hypothesis we grew HEK cells that express high levels of p53 in a cell culture centrifuge for 24 hours. We then compared p53 levels from cells cultured at 25 times unit gravity (25g), to cells cultured at normal gravity (1g). Western blot analysis of p53 levels reveal that cells exposed to 25g have depleted levels of p53 as compared to cells cultured in 1g. Densitometry analysis shows a 45% decrease in p53 levels in cells exposed to 25g. Culture of primary osteoblasts at 25g for 24h also increased cell proliferation 1.5 fold relative to 1g, in an integrin and extracellular matrix-dependent manner. These results indicate that mechanical cues regulate p53 levels in a bi-directional manner. Loss of mechanical input results in an accumulation of p53 while the present study shows p53 depletion in response to increased mechanostimulation. The role of gravity-induced mechanical forces in regulating p53 levels via a matrix/integrin pathway (Ilic et al. 1998, Almeida et al. 2000) suggests a possible mechanism for the regulation of cell proliferation by mechanical stimuli. Supported by NASA-00-OBPR-01.

## Mitosis & Meiosis IV (2292-2321)

2292

**γ-Tubulin Localization in the Mitotic Spindle**

J. Ho, C. D. Yankel, S. A. Endow; Cell Biology, Duke University Medical Center, Durham, NC

γ-Tubulin is thought to function in microtubule nucleation and has been localized to microtubule organizing centers (MTOCs) in a variety of organisms. To investigate the localization of γ-tubulin in the mitotic spindle, we created transgenic *Drosophila* lines that express γ-tubulin-GFP. The *γ-tubulin-gfp* lines show no detectable mutant effects due to the gene insert. Remarkably, γ-tubulin-GFP not only localizes to the centrosome, but also localizes to the spindle in live *Drosophila* embryos during the syncytial blastoderm stages of early embryonic development. γ-Tubulin-GFP does not fully decorate the spindle, but instead appears to be associated with a subset of spindle microtubules in a pattern that resembles the distribution of kinetochore microtubules. γ-Tubulin-GFP fluorescence showed more rapid loss during photobleaching than the spindle-and centrosome-associated microtubule motor protein Ncd, indicating that γ-tubulin differs greatly in its binding to the centrosomes compared to Ncd. γ-Tubulin-GFP also reassociated with centrosomes less rapidly than Ncd after photobleaching. Photobleaching of half or full spindles did not appear to affect cellular division in the *γ-tubulin-gfp Drosophila* line. Our results suggest that γ-tubulin is bound to a substructure of the spindle that may represent the elusive spindle matrix.

2293

**Determinants of Cohesin Localization**

J. L. Gerton,[1,2] C. Bausch,[1] S. Noone,[1] K. Zueckert-Gaudenz,[1] C. Seidel[1]; [1]Stowers Institute for Medical Research, Kansas City, MO, [2]Department of Biochemistry and Molecular Biology, University of Kansas Medical School, Kansas City, KS

In eukaryotic cells, cohesion allows sister chromatids to biorient on the metaphase plate and holds sister chromatids together until they separate into daughter cells during mitosis. Cohesion is mediated by the cohesin protein complex. We have used chromatin immunoprecipitation coupled with microarray analysis (ChIP chip) to produce a genome-wide description of cohesin association with mitotic chromosomes in *S. cerevisiae.* Although the pattern of association is highly reproducible, no consensus binding sequence could be identified. However, we observed a very strong correlation between cohesin sites and regions between convergent transcription units. We have begun to explore the mechanistic relationship between cohesin association and transcription. Transcript elongation into sites with which cohesin is associated results in the disassociation of cohesin. Interestingly, once transcription is halted, cohesin reassociates with its original sites, independent of DNA replication. These results and others indicate that cohesin association with chromosomes is both flexible and dynamic. Our results are most consistent with a model in which cohesin localization is determined by chromatin state.

2294

**Mutual Inhibition of Separase and Cdk1 by Two-Step Complex Formation**

O. Stemmann, I. H. Gorr, D. Boos; Molecular Cell Biology, Max-Planck-Institute of Biochemistry, Martinsried, Germany

Stable maintenance of genetic information requires chromosome segregation to occur with high accuracy. Anaphase is triggered when ring-shaped cohesin is cleaved by separase, a protease regulated by association with its inhibitor securin. Dispensability of vertebrate securin strongly suggests additional means of separase regulation. Indeed, sister chromatid separation but not securin degradation is inhibited by constitutively active cyclin-dependent kinase 1 (Cdk1) and can be rescued solely by preventing phosphorylation of separase. We demonstrate that Cdk1-dependent phosphorylation of separase is not sufficient for inhibition. In a second step Cdk1 stably binds phosphorylated separase via its regulatory cyclin B1 subunit. Complex formation results in inhibition of both protease and kinase, and we show that vertebrate separase is a direct inhibitor of Cdk1. This unanticipated function of separase is negatively regulated by securin but independent of separase's proteolytic activity.

2295

**The Stress-activated G2 Antephase Checkpoint is a General Feature of Normal But Not Transformed Cell Lines**

A. V. Mikhailov,[1,2] C. L. Rieder[1,2]; [1]Molecular Medicine, Wadsworth Center of the NYS Dept of Health, Albany, NY, [2]Biomedical Sciences, SUNY School of Public Health, Albany, NY

Animal cells contain a cell cycle checkpoint during G2, centered on the p38-stress-activated protein kinase, that delays entry into mitosis in response to various stresses that could interfere with the proper segregation of the genome. We have examined the functionality of this checkpoint in normal and cancerogenic (transformed) cells by exposing them during chromosome condensation to low concentrations of the p38 activator, anisomycin. We confirmed p38 activation with p38 phospho-specific antibodies, and used progress through antephase (the first signs of chromosome condensation to the commitment to mitosis near nuclear envelope breakdown) as a visual cue of the cells' progression through G2. In response to anisomycin treatment during antephase, primary and telomerase-immortalized epithelial and fibroblastic cells decondensed their chromatin and returned to G2. However, 4-5 hrs later they then completed a normal antephase and entered mitosis. In contrast, many (e.g., Hela, dormant Hep3, tumorigenic Hep3), but not all (e.g., U2OS), transformed cells progressed through antephase and entered mitosis with normal timing in spite of strong p38 activation. In these cells the defect in the antephase checkpoint must therefore lie downstream of the p38 kinase. Entering mitosis under stressful conditions can lead to chromosome nondisjunction/damage, and to the loss or gain of whole chromosomes or chromosome parts. We propose that defects in the antephase checkpoint in early stage tumors lead to genomic instability. Further, checkpoint functionality may be restored in advanced cancers to stabilize genetic changes and avoid cell death during chemotherapy.

2296

**CENP-E Promotes Chromosome Congression By Lateral Transport Along Microtubule Fibers**

M. A. Lampson,[1] P. Hergert,[2] A. Khodjakov,[2] T. M. Kapoor[1]; [1]Laboratory of Chemistry and Cell Biology, Rockefeller University, New York, NY, [2]Wadsworth Center, New York State Department of Health, Albany, NY

During cell division chromosomes move to the center of the mitotic spindle (congress) as they correctly attach to spindle microtubules and align at the metaphase plate. In mammalian cells most chromosomes are located between the spindle poles after nuclear envelop breakdown and rapidly form attachments to both spindle poles. A few chromosomes cannot readily achieve proper attachments due to their position and are transported to one spindle pole, where they delay anaphase by activating the mitotic spindle checkpoint. The precise steps required for these pole-associated chromosomes to congress and achieve bi-orientation remain unknown. We developed an assay, using reversible cell-permeable small molecule kinesin and kinase inhibitors, to accumulate chromosomes near the pole and examine their congression. Correlative light and electron microscopy allowed a detailed analysis of the early steps of this process. Cells were imaged live to identify chromosomes in the process of congressing from the pole, then fixed, relocated, and analyzed by serial section EM. We find that the leading kinetochores of congressing chromosomes are not attached to the ends of microtubule fibers, as would be expected if bi-orientation preceded congression, but instead are attached to the sides of the fibers. This finding suggests that a microtubule motor located at the kinetochore may drive this congression process by walking along the sides of microtubule fibers. When the mitotic kinesin CENP-E is depleted by RNAi, chromosomes remain at the pole and fail to congress in our assay. The formation of syntelic chromosome attachments in CENP-E-depleted cells indicates that the congression defect does not result from a failure in microtubule attachment alone. We propose that CENP-E promotes congression of chromosomes at the spindle pole by lateral transport along microtubule fibers.

2297

**Identification of Drosophila Spindly, a Novel Factor Required for Cellular Morphology and Mitotic Progression**

E. R. Griffis, N. Stuurman, R. D. Vale; HHMI/CMP, UCSF, San Francisco, CA

We have recently completed a morphology screen in Drosophila S2 cells using a library of over 7200 dsRNAs. Among the novel candidates that we found in the screen, was a protein whose depletion produced defects in lamellar actin and long microtubule rich projections. The sequence of this protein does not reveal obvious homologues in other organisms, and based upon the unusual cell projections after RNAi, we call this protein Spindly. When GFP-tagged Spindly was expressed in S2 cells, we found that it localizes to microtubule tips, to the actin-rich lamella, and on the kinetochores of mono-oriented and unaligned chromosomes. The latter localization suggests a role for spindly in mitosis, and indeed, spindly RNAi results in an elevated mitotic index and abnormal spindles. In live cells, we have observed that as chromosomes achieve biorientation and move towards the metaphase plate, the level of spindly on kinetochores decreases, and a new population of Spindly appears on spindle microtubules and spindle poles. By closely evaluating GFP-Spindly movement during mitosis, we were able to discern discreet particles of the protein tracking along microtubules from kinetochores to the spindle pole. Interestingly, we find that depletion of cytoplasmic dynein stops the mitotic movements of GFP-Spindly, while depletion of the Rough Deal (Rod) checkpoint protein prevents the association of Spindly with kinetochores. Conversely, when we deplete Spindly we have observed that Rod accumulates to high levels on all kinetochores, similar to what is seen after dynein depletion, a likely explanation of the elevated mitotic index. Additionally, codepletion of spindly together with other mitotic factors produces phenotypes similar to those seen after dynein codepletion. From these data, we hypothesize that spindly is a novel dynein or dynactin cofactor.

2299

**A Marine Natural Product that Blocks Mitosis but Does Not Target Tubulin**

M. K. Jung,[1] K. Stewart,[2] K. Yamada,[3] H. Kigoshi,[4] K. Suenaga,[4] C. E. Oakley,[5] B. R. Oakley,[5] E. Hamel[6]; [1]Screening Technologies Branch, SAIC-Frederick, Inc., Frederick, MD, [2]Screening Technologies Branch, National Cancer Institute at Frederick, Frederick, MD, [3]Graduate School of Science, Nagoya University, Nagoya, Japan, [4]Department of Chemistry, University of Tsukuba, Tsukuba, Japan, [5]Department of Molecular Genetics, The Ohio State University, Columbus, OH, [6]Toxicology and Pharmacology Branch, National Cancer Institute at Frederick, Frederick, MD

Although anti-mitotic agents have generally targeted tubulin, we want to identify new anti-mitotic compounds with targets other than tubulin and to use these agents to identify cellular components involved in mitotic control and regulation. Mitosis is a validated anti-cancer target. Besides the clinically important vinca alkaloids and taxoids, current clinical trials include new inhibitors of tubulin, aurora kinases and the kinesin spindle protein, KSP or Eg-5. In a screen to identify compounds that prevent the progression of cells through mitosis, we discovered that aplyronine A, a marine natural product cytotoxic to tumor cells, arrests MCF-7 breast cancer cells in mitosis at sub-nanomolar concentrations. A macrolide isolated from the sea hare *Aplysia kurodai*, aplyronine A does not affect *in vitro* assembly of brain tubulin. Exposure of tissue culture cells to aplyronine A disrupts mitotic spindles, and the resulting spindle morphology differs from what is seen after exposure to classical anti-microtubule agents. The spindle morphology also differs from that seen in cells exposed to inhibitors of aurora kinase or Eg-5. Potential aplyronine A targets include structural components of the mitotic apparatus, centrosomal components, and signaling molecules. There is evidence that aplyronine A interacts with a component of the fungal spindle pole body (the centrosome equivalent), since it supports the growth of a conditionally lethal γ tubulin mutant of the filamentous fungus *Aspergillus nidulans*. Supported by NIH contract NO1-CO-12400 and NIH grant GM31837.

2300

**Molecular Composition and Cellular Dynamics of a Mitotic Checkpoint Complex**

J. V. Shah,[1] Z. Wang,[2] G. J. P. Kops,[3] I. McLeod,[4] J. R. Yates,[4] M. W. Berns,[5] D. W. Cleveland[6]; [1]Systems Biology, Harvard University, Boston, MA, [2]Developmental Biology, University of California at Irvine, Irvine, CA, [3]Experimental Oncology, UMC Utrecht, Utrecht, The Netherlands, [4]Scripps Research Institute, La Jolla, CA, [5]Beckman Laser Institute, University of California at Irvine, Irvine, CA, [6]Ludwig Institute for Cancer Research, University of California at San Diego, La Jolla, CA

The mitotic checkpoint guards against chromosome loss by delaying anaphase onset until each and every chromosome is bioriented on the mitotic spindle. This pathway acts through the production of an inhibitory signal from unattached kinetochores that blocks the ubiquitin ligase activity of the anaphase-promoting complex (APC/C). The action of this inhibitory signal is thought to either sequester a mitotic APC/C regulator, Cdc20, to prevent APC/C activity or modulate the APC/C in the context of Cdc20 to prevent its activity against anaphase substrates. Here we demonstrate that within the cytoplasm of mitotic cells with unattached chromosomes Mad2, a central checkpoint protein, forms a robust complex with the APC/C that includes Cdc20 and other checkpoint proteins as detected by mass spectrometry. In vivo measurements of the Mad2-APC/C complex made in living cells, using fluorescence correlation spectroscopy, demonstrate that this complex is silenced by the dissolution of this inhibitory complex below a threshold concentration that permits the transition to anaphase. In addition, this complex is present in interphase, potentially acting as a pre-made anaphase inhibitor upon entry into mitosis. The combined proteomic and live cell spectroscopy approach reveals both composition and temporal kinetics and will prove to be a useful methodology for systematic dissection of signaling pathways.

2301

**A Conditional Mutation of mMps1 Is Embryonic Lethal and Induces Tumors in T Cells**

S. Z. Xie, P. K. Sorger; Biology, Massachusetts Institute of Technology, Cambridge, MA

The mitotic spindle checkpoint ensures accurate chromosome segregation by delaying anaphase onset until all kinetochores have achieved bipolar attachment to the spindle apparatus. Mps1 is a conserved serine/threonine protein kinase essential for proper chromosome segregation. Abrogation of the mammalian mitotic checkpoint through RNAi depletion of hMps1 causes various mitotic errors including chromosomal instability (CIN). A strong correlation between cancer and CIN has made the mitotic checkpoint proteins attractive candidates as tumor suppressors. Few such spindle checkpoint mutations have been found in human cancers, however, and it is possible that complete loss of checkpoint function might result in mitotic lesions that are too severe for tumor development. To determine if the generation of partial-loss of function mutations in checkpoint proteins might result in CIN and cancer in mice, a conditional FLOX (flanked by LoxP) allele of Mps1 was constructed. This allele, Mps1Δ1, removes a highly conserved Mps1 domain that retains proper kinetochore localization but that is lethal by embryonic day 10.5. Since the immune system is both highly proliferative and non-essential, Mps1Δ1-FLOX mice were crossed to a transgenic line expressing a T-cell specific Lck-Cre gene We find that mice carrying a homozygous Mps1Δ1 allele in their T cells have higher than normal number of lymphomas. Intriguingly, 100% of mice combining a hetereozygous deletion of p53 with Mps1Δ1/Mps1Δ1 develop thymomas within 3.5 months. Cell lines derived from these

primary tumors are highly aneuploid but still arrest in mitotis following treatment with the microtubule destabilizing drug nocodazole. We conclude that partial loss of Mps1 creates a CIN phenotype that is greatly exacerbated by p53 haplo-insufficiency and strengthens our recent finding that complete loss of p53 can suppress the lethality associated with deletion of the Mad2 gene in murine fibroblasts.

2302

**Investigating the Spindle Checkpoint in Early *C elegans* Embryos**

A. Essex,[1] A. Dammermann,[2] J. Monen,[1] K. Oegema,[1] A. Desai[1]; [1]Biomedical Sciences, University of California, San Diego/Ludwig Institute for Cancer Research, San Diego, CA, [2]Ludwig Institute for Cancer Research, San Diego, CA

The spindle checkpoint delays anaphase onset until all chromosomes are properly attached to the spindle. Previous studies have shown that BUB-1 is essential for checkpoint activation and have also identified checkpoint-independent functions for this conserved kinase. We are utilizing the early C. elegans embryo to investigate BUB-1 function and to analyze the relationship between kinetochore structure and checkpoint activation. Depletion of BUB-1, but not other checkpoint pathway proteins, results in premature separation of spindle poles in the first embryonic mitosis, indicative of an inability to form kinetochore-microtubule attachments capable of resisting forces pulling on the spindle poles. However, spindle poles move back towards the metaphase plate just prior to anaphase onset, suggesting that this defect is temporal rather than absolute. Anaphase bridges are frequently observed for oocyte-derived chromosomes (which underwent meiosis in BUB-1-depleted cytoplasm), suggesting a role for BUB-1 in meiotic segregation. Consistent with this idea, BUB-1 localizes to the axis of cohesion loss during both meiotic divisions, in addition to co-localizing with outer kinetochore proteins on the chromosome surface. To develop a robust functional assay for checkpoint activation in C. elegans, we have exploited the consequences of depleting ZYG-1, a kinase required for centrosome duplication. Following ZYG-1 depletion, the first mitotic division is normal, but monopolar spindles form in both daughter cells in the second division. The presence of monopolar spindles results in a striking delay in the metaphase-anaphase transition, assessed using chromosome decondensation and onset of cortical contractility in live imaging data. Double depletion of ZYG-1, and Mad1/Mad2 does not affect first division timing but abolishes the delay observed in the second division. We are using this assay to analyze the role of BUB-1, other checkpoint pathway proteins, and conserved outer kinetochore components in checkpoint activation.

2303

**An Enhancer of Myosin Speeds Up Anaphase Chromosome Movements**

L. Fabian, J. Troscianczuk, A. Forer; Biology Department, York University, Toronto, ON, Canada

Inhibitors of actin/myosin block anaphase movements in insect spermatocytes (Forer & Pickett-Heaps 1998, Chromosome Research 6: 533-549; Fabian & Forer *in press*, Protoplasma). We studied the effects of CalyculinA (CalA), which e*nhance*s myosin function: by blocking dephosphorylation by myosin light chain phosphatase (MLCP), CalA causes myosin to stay activated. When CalA is added to anaphase crane-fly spermatocytes the chromosomes speed up (5 times faster); they don't form new nuclei in late anaphase/telophase but they move back toward the equator. When CalA is added during metaphase, chromosomes move faster during anaphase, the same as in anaphase treated cells. When CalA is added during prometaphase, chromosomes move fast up and back in the spindle, jiggle and rotate. In order to confirm that CalA action is on myosin, we treated cells with Y-27632 (a Rho-K inhibitor) and CalA, or with BDM (an inhibitor of myosin motor activity) and CalA. Y-27632 ordinarily slows anaphase movements (Fabian & Forer, *in press*, Protoplasma). We treated anaphase cells with CalA, and then we added Y-27632 (in CalA): the chromosomes still sped up. Y-27632 added before CalA caused chromosomes to slow, as expected, but when CalA (in Y-27632) was added to the same cells, the previously slowed chromosomes sped up, reaching initial speeds or higher. Telophase chromosomes also moved backwards. BDM added to CalA-treated cells, after the chromosomes sped up, caused the chromosomes to slow or to stop. All these results are consistent with the effects of CalA being on myosin dephosphorylation. Immunofluorescence studies show that CalA affects the distribution and organization of spindle microtubules, spindle actin, cortical actin and putative spindle matrix proteins skeletor and titin. Our results suggest an important role for myosin during anaphase.

2304

**Computational Model of Chromosome Motility in *Drosophila* Embryos**

G. Civelekoglu-Scholey,[1] D. J. Sharp,[2] A. Mogilner,[1] J. M. Scholey[1]; [1]Center for Genetics and Development, University of California, Davis, Davis, CA, [2]Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY

Chromosome motility during metaphase and anaphaseA is coupled to kinetochore microtubule (kMT) dynamics but the precise mechanism of this coupling is poorly understood. For example, kMTs undergo dynamic instability at their kinetochore bound plus ends but at the same time display persistent poleward flux, driven in part by kinesin-13-dependent depolymerization at their minus ends. In the Drosophila embryo, spindle microtubules (MT) turn over at a remarkably rapid rate giving rise to a FRAP half-time of ~5 sec as they rapidly flux polewards at ~ 0.05 μm $sec^{-1}$ (Brust-Mascher et al. 2004). AnaphaseA chromatid-to-pole motility rate is also remarkably fast (~ 0.1 μm $sec^{-1}$) and depends upon a combined flux-pacman mechanism (Rogers et al. 2004). Here we develop a force-balance model of chromosome motility based on the antagonistic and complementary action of polymer ratchets and motor-generated forces that are thought to operate at kinetochores and spindle poles in *Drosophila* embryo, coupled to kMT plus end dynamics regulated through forces and MT-based motors (Rogers et al. 2004, Sharp et al. 1999, Yucel et al. 2000, Rogers et al. 2001). The model shows that (i) both the metaphase positioning and rapid anaphaseA chromosome motility can be described in a unified framework, based on the action of motor proteins coupled to MT dynamics; (ii) high dynein activity at the kinetochore is required to ensure sustained metaphase kinetochore-MT attachment if the flux rate and MT dynamics are high as in *Drosophila*; (iii) high dynein activity at the kinetochore also results in rapid anaphaseA rates and dampens the metaphase chromosome oscillations. Finally we address the generality of the proposed *Drosophila* pacman motor (Mennella et al., 2004) and consider the minimal structural and molecular properties of kinetochores and MTs that correlate with chromosome motility events observed in different organisms.

2305

**Condensin is Essential to Prevent Premature Chromosome Decondensation in Anaphase**

P. Vagnarelli, D. F. Hudson, W. Earnshaw; Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom

The mechanisms underlying the establishment of mitotic chromosome condensation and its maintenance until the end of mitosis remain two of the classic unanswered questions of cell biology. It has recently been proposed that two protein complexes called condensin I and II are important for this condensation, and are essential for topoisomerase II to separate sister chromatids during anaphase. We have used a gene targeting analysis to determine the essential function of condensin in chicken DT40 cells. Our results differ significantly from those predicted by present models of condensin function. Condensin is not essential for mitotic chromosomes to adopt an orderly higher-order structure, for normal cohesin dynamics or for sister chromatid separation during anaphase. Neither is it essential for topo II function at centromeres or on the chromosome arms. Instead, condensin is essential for vertebrate chromosomes to remain condensed throughout anaphase. In condensin-depleted cells chromatids abruptly decondense while still moving during anaphase B. However, chromatids can remain condensed and segregate normally provided that CDK levels are kept high during mitotic exit. Our results identify a new activity, RCA (regulator of condensation in anaphase), that cooperates with condensin to maintain mitotic chromosome condensation in anaphase.

2306

**Mps3p, a Nuclear Membrane SUN-Domain Protein, Mediates Telomere Repositioning to Form the Meiotic Bouquet**

C. Y. Lee, M. N. Conrad, J. L. Wilkerson, M. E. Dresser; Molecular, Cell and Developmental Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK

The meiotic chromosome bouquet forms when telomeres associate with the nuclear envelope and cluster adjacent to the centrosome. The bouquet is well-conserved, indicating an important role in meiosis, but the molecular mechanisms involved are poorly understood. In *Saccharomyces cerevisiae*, bouquet formation requires the meiosis-specific telomere-binding protein Ndj1p. Ndj1p interacts in two-hybrid assays with Mps3p, an essential nuclear envelope protein and component of the spindle pole body (the centrosome-equivalent). We have identified and deleted an Ndj1p interaction domain of Mps3p (amino acids 2-64). *Mps3*$^{\Delta 2\text{-}64}$ mutants are fully viable but defective in bouquet formation. Furthermore, *ndj1Δ*, *mps3*$^{\Delta 2\text{-}64}$ and the *ndj1Δ mps3*$^{\Delta 2\text{-}64}$ double mutant all have similarly delayed chromosome pairing and elevated chromosome nondisjunction. These results identify *MPS3* as the second gene in the *NDJ1* pathway of bouquet formation and establish a specific, direct interaction between telomeres and the spindle pole. During meiotic prophase, Mps3p localizes to the spindle pole body but also accumulates and colocalizes with clumps of telomeres along the nuclear envelope. This colocalization is dependent on Ndj1p, arguing that Ndj1p connects or stabilizes the interaction between telomeres and Mps3p. As a member of the SUN family of proteins implicated in nucleus-cytoskeleton interactions, Mps3p may link telomeres to cytoplasmic structures that provide poleward forces during bouquet formation. Htz1p, a histone-like chromatin protein, also interacts with Mps3p in two-hybrid assays. We find that *htz1Δ* has no delay in homologous chromosome pairing but nevertheless has elevated nondisjunction. Early evidence suggests that *htz1Δ* is defective in bouquet formation, suggesting that timely homologous chromosome pairing may not require formation of a stable bouquet.

2307

**The Human Ubiquitin Conjugating Enzyme, UBE2E3, is Required for the Completion of Cytokinesis**

K. M. Ross,[1] K. S. Plafker,[1] A. L. Oldham,[1] G. J. Gorbsky,[2] S. M. Plafker[1]; [1]Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, [2]Molecular, Cell, and Developmental Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK

Cell cycle progression is governed by the coordinated activities of protein kinases and the ubiquitin proteolytic system. This interplay of phosphorylation and ubiquitylation drives unidirectional movement through and between each cell cycle stage and is essential for maintaining genomic stability. Experiments in yeast as well as reconstitution studies using oocyte and mammalian somatic cell extracts have identified several key ubiquitin enzymes that control cell division. However, considerably less in vivo evidence has been generated in mammalian cells to determine if other ubiquitin enzymes might also be involved. Here we report on our studies of the human ubiquitin conjugating enzyme, UBE2E3. We have found by RT-PCR and western blot analyses that this evolutionarily conserved enzyme is expressed in a variety of organs including brain, liver, lung, and kidney and that the levels of UBE2E3 appear to be constant throughout the cell cycle. We have used RNA interference in HeLa cells to investigate the function of the enzyme and demonstrated that depletion of UBE2E3 results in multi-nucleated cells, pleomorphic nuclei, decreased cell proliferation, changes in cell shape, and cell death. Time-lapse video microscopy revealed that loss of UBE2E3 leads to a failure of cells to resolve the cytokinetic midbody. The multi-nucleated cells result from retraction of the midbody and fusion of the daughter cells following the failure of midbody abscission. Together, these data demonstrate that UBE2E3 is expressed in numerous tissues and functions to maintain genomic stability at least in part by promoting abscission of the midbody during the terminal stage of cytokinesis.

2308

**Phospholipase D-Generated Phosphatidic Acid and GGAs in *Saccharomyces cerevisiae* Sporulation**

R. Mendonsa,[1] J. Engebrecht[2]; [1]Cell and Developmental Biology Graduate Group, Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA, [2]Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA

Gametogenesis is an essential, conserved process in all organisms that reproduce sexually. In budding yeast sporulation, meiosis must be tightly coupled to spore morphogenesis to ensure the production of viable gametes. Spindle pole bodies associated with meiotically dividing nuclei form thickened plaques next to the nuclear surfaces and serve as fusion sites of Golgi-derived vesicles, leading to the formation of the spore plasma membrane precursor, the prospore membrane. The forming prospore membrane remains closely associated with nuclei while additional spore wall materials are deposited in successive layers, and eventually the nuclei are completely enclosed as individual spores within the original plasma membrane. The activity of yeast phospholipase D, or *SPO14*, has been shown to be essential for prospore membrane formation. Spo14p catalyzes the cleavage of phosphatidylcholine into phosphatidic acid (PA) and choline. PA is thought to be involved in membrane trafficking events, although the mechanism of its action in spore morphogenesis is unknown. Using an *in vitro* proteomics approach to find downstream effectors of Spo14p-generated PA, we recently identified an interaction between PA and both Gga1p and Gga2p, which are multi-domain proteins involved in ubiquitinated protein trafficking from the trans-Golgi network. We constructed isogenic *gga* deletion strains and observed reduced sporulation in the double *gga* mutant compared to wild-type. We also tested double *gga* mutant spores for ether sensitivity, as vegetative cells and aberrant spores are ether-sensitive while wild-type spores are ether-insensitive, and the double *gga* mutant was shown to be sensitive to ether exposure. These observations suggest that GGAs may be downstream effectors of Spo14p-generated PA and are involved in the production of viable spores.

2309

**Homology-Independent Centromere Coupling and Initiation of Synaptonemal Complex Formation during Meiosis in Budding Yeast**

T. Tsubouchi, S. Roeder; Yale University, New Haven, CT

Segregation of chromosomes at the reductional division of meiosis depends on a series of interactions between homologous chromosomes, including pairing, assembly of the synaptonemal complex (SC) and genetic recombination. These events ultimately lead to the formation of chromatin bridges between homologs that ensure their segregation to opposite poles at the first meiotic division. Through cytological studies, we have discovered a process in meiotic cells of budding yeast, in which chromosomes become joined together in pairs at their centromeres via an SC protein (Zip1). In a mutant defective in the initiation of meiotic recombination, all centromeres are coupled, but almost all couples involve nonhomologous chromosomes. During meiosis in wild-type diploids, centromere couples are initially nonhomologous and then undergo switching until all couples involve homologs. We have also discovered that regions of SC assembled early in meiosis are often centromere associated. SC assembly requires the so-called the synapsis initiation complex (SIC). Our data from immunolocalization studies suggest that the SC often forms unidirectionally, with the SIC at the leading edge of the polymerizing Zip1. We found that Zip3, a component of the SIC, localizes to centromeres at an early stage of prophase in a partially Zip1-dependent manner, prior to the loading of two other SIC components (Zip2 and Zip4). Thus, loading of the SICs at centromeres is preceded by and is at least partially dependent on Zip1 loading. We propose that Zip1-dependent centromere coupling facilitates homolog pairing and ensures synapsis initiation on every pair of chromosomes.

2310

**Mer2 Forms a Complex with Rec114 and Mei4 to Form Meiotic Double-strand Breaks**

J. Li,[1] G. W. Hooker,[1] S. Roeder[2,3]; [1]Molecular, Cellular & Developmental Biology, Yale University, New Haven, CT, [2]Molecular, Cellular & Developmental Biology, Department of Genetics, Yale University, New Haven, CT, [3]Howard Hughes Medical Institute, New Haven, CT

In budding yeast, at least ten proteins are absolutely required for the formation of meiotic double-strand breaks (DSBs). A combination of cytological studies and biochemical analysis was used to investigate the functions of these proteins, with particular emphasis on Mer2. Mer2 protein localizes to meiotic chromosomes, forming distinct foci from zygotene to pachytene, with foci maximally abundant in zygotene. Localization of Mer2 is independent of DSBs and the other proteins required for DSB formation. Mer2 is phosphorylated specifically in meiosis and this modification is independent of a number of known kinases (Mek1, Mec1 and Tel1) and occurs in the absence of DSB formation. Mer2 associates with itself, as demonstrated by the yeast two-hybrid system and biochemical experiments. Previous work in the field has shown that there are two separate complexes involved in DSB formation: Spo11/Rec102/Rec104 and Mre11/Rad50/Xrs2. Here we show that Mer2 forms a complex with Mei4 and Rec114. First, cytological experiments show that Mei4, Rec114 and Mer2 partially colocalize with one another. Second, Mei4 and Rec114 interact in the yeast two-hybrid system. Lastly Mer2, Mei4 and Rec114 coimmunoprecipitate with one another from meiotic cell extracts. The Mer2/Mei4/Rec114 complex is independent of the other two complexes. Mer2 does not colocalize with Mre11, Spo11 or Rec102 and Mer2 does not coimmunoprecipitate with Rec102. We propose that Mer2, Rec114 and Mei4 form an independent complex that is involved in DSB formation.

2311

**Meiotic Cohesin Modulates Chromosome Compaction During Meiotic Prophase**

D. Ding, Y. Hiraoka; Kansai Advanced Research Center, Kobe, Japan

During the mitotic cell cycle the regulated cohesion and segregation of sister chromatids is achieved by the mitotic cohesin complex, and upon entering meiosis, this mitotic cohesin complex is replaced by the meiotic cohesin complex which choreographs the meiosis-specific chromosomal events. The meiotic cohesin, Rec8, is required for sister chromatid cohesion and homologous recombination. By directly measuring chromosome compaction in living cells of the fission yeast Schizosaccharomyces pombe, we found an additional role for cohesin in the compaction of chromosomes during meiotic prophase. In the absence of Rec8, chromosomes were loosened 2 fold when compared with wild-type cells, while the loss of the cohesin-associated protein, Pds5, resulted in Rec8-dependent over-compaction. Both homologous pairing and recombination were defective in the absence of either Rec8 or Pds5. Pds5 also demonstrated a role in maintaining sister chromatid cohesion and in reductional chromosome segregation. In the absence of Pds5, the Rec8 binding on chromosome was decreased, indicating that Pds5 modulate the association of Rec8 with meiotic chromosomes. Thus, the cohesins mediate fundamental changes in global chromatin structure that promote correct meiotic progression.

2312

**Novel Protein Components Bqt1 and Bqt2 Regulate the Transition Between Mitotic and Meiotic Organization of Chromosomes**

Y. Chikashige, Y. Hiraoka; Kansai Advanced Research Center, Kobe, Japan

In many organisms, meiotic chromosomes are bundled at the telomere to form a ‘bouquet’ arrangement. The most striking example of such chromosome reorganization is observed in the fission yeast S. pombe. In this organism, centromeres are clustered at the spindle-pole body (SPB) during the mitotic cell cycle, and telomeres form a cluster at the SPB during meiotic prophase; this clustering of telomeres is important for progression of meiosis, promoting homologous chromosome pairing and recombination during meiosis. However, direct interactions have not been found between the telomere and the SPB. As meiotic telomere clustering is induced by the mating pheromone response, we selected candidate genes that are expressed specifically under mating pheromone signaling based on gene expression profiles in DNA microarray data. To find telomere-SPB connectors, we disrupted each one of the selected 83 genes in S. pombe. All of these gene-disrupted strains were viable in mitotic growth as expected from meiosis-specific expression of the gene. These strains were microscopically observed for telomere clustering in meiotic prophase. Such a genome-wide search found two novel proteins, Bqt1 and Bqt2 (named for ‘bouquet’ formation), which connect Rap1 (a telomere protein) to Sad1 (an SPB protein) when mating pheromone signaling is induced in meiosis. Neither Bqt1 nor Bqt2 alone functions as a connector, but the two proteins together form a bridge between Rap1 and Sad1. Significantly, when both Bqt1 and Bqt2 are ectopically expressed in mitotic cells, they form a bridge between Rap1 and Sad1. As mitotic centromere-SPB connectors are resolved under mating pheromone signaling in meiosis (Asakawa et al., 2005, Mol. Biol. Cell, 16, 2325-2338), expression of Bqt1 and Bqt2 under mating pheromone signaling regulates the transition between mitotic and meiotic chromosome organization.

2313

**Dissociation of the Nuf2-Ndc80 Complex Releases Centromeres from the Spindle-Pole Body under Mating Pheromone Signaling in Fission Yeast Meiosis**

H. Asakawa,[1] A. Hayashi,[1] T. Haraguchi,[1,2] Y. Hiraoka[1,2]; [1]Cell Biology Group and CREST Research Project, Kansai Advanced Research Center, National Institute of Information and Communications Technology, Kobe, Japan, [2]Graduate School of Science, Osaka University, Toyonaka, Japan

In the fission yeast *Schizosaccharomyces pombe*, centromeres remain clustered at the spindle-pole body (SPB) during mitotic interphase. In contrast, during meiotic prophase centromeres dissociate from the SPB. Nuf2 is a centromere protein known as a component of Ndc80/Hec1 complex in eukaryotes. We previously reported the behavior of centromeres correlates with Nuf2 localization in *S. pombe*: Nuf2 is located at the centromere in the nucleus throughout the mitotic cell cycle, but is not observed during meiotic prophase when the centromeres are separated from the SPB. Here we examine the behavior of centromere proteins in living meiotic cells of *S. pombe*, and show that the *S. pombe* Nuf2-Ndc80 complex proteins (Nuf2, Ndc80, Spc24 and Spc25) always located at the centromere during mitotic cell cycle, while they disappear from the centromere in meiotic prophase. Nuf2 interacts with other centromere protein Mis12, but not with Mis6. The Mis12 dissociates from centromeres during meiotic prophase; however Mis6 remains throughout meiosis, suggesting distinct roles of centromere subcomplexes in meiosis. When cells are induced to meiosis by inactivation of Pat1 kinase (a key negative regulator of meiosis), centromeres remain associated with the SPB during meiotic prophase. However, inactivation of Nuf2 by a temperature-sensitive mutation causes the release of centromeres from the SPB in *pat1* mutant cells, suggesting that the Nuf2-Ndc80 complex connects centromeres to the SPB. We further found that removal of the Nuf2-Ndc80 complex from the centromere and centromere-SPB dissociation are caused by mating pheromone signaling. Since *pat1* mutant cells also show aberrant chromosome segregation in the first meiotic division and this aberration is compensated by mating pheromone signaling, dissociation of the Nuf2-Ndc80 complex may be associated with remodeling of the kinetochore for meiotic chromosome segregation.

2314

**Alleles of AFD1 Unravel Stage-Specific Activities of REC8 during Meiosis**

I. N. Golubovskaya, L. Timofejeva, O. Hamant, W. Cande; MCB, UC Berkeley, Berkeley, CA

Maize has four rad21/rec8 genes. Afd1 (absence of first division 1), closest in homology to rec8, controls leptotene chromosome establishment and sister chromatid cohesion (SCC). In two null alleles (afd1-1, afd1-2) an equational segregation of sisters occurs during M I due to a failure of both arm and centromere SCC. AFD1 protein is recruited early, before the leptotene stage, and localizes to the lateral elements of zygotene and pachytene chromosomes. AFD1 is progressively removed from the chromosomes arms during diplotene/diakinesis and is not detected after metaphase I. In two weak afd1 alleles, afd1-3 and afd1-4, leptotene chromosomes are present, however, no homologous pairing occurs. The formation of synaptic structures depends on the severity of the allele: only short condensed synaptic structures (15/nucleus) are observed in afd1-1 and afd1-2, whereas in afd1-3 and afd1-4, their number is increased by 5-10 fold, and fragments of axial elements are visible. In the weak alleles at anaphase I, segregation of chromosomes is neither equational nor reductional, as the cell contains chromatids and X-shape chromosomes, showing that loss of SCC occurs at later in the weak afd1 alleles. Molecular analysis of the weak alleles showed that a wild-type cDNA is produced but at a lower level than in the wild-type. Our analysis shows that afd1 allelic severity impacts differentially leptotene chromosome establishment, and the extent of synapsis and SCC. This suggests that meiosis requires a stage-specific AFD1 activity and that conversely, the meiocyte carefully monitors the activity of AFD1 during meiosis.

2315

**2-Dimensional Gel Electrophoresis Screen of Global Changes in Protein Expression During Progesterone Induced Maturation of *Xenopus laevis* Oocytes**

L. C. Berger, A. Wilde; Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada

The *Xenopus laevis* oocyte is an established model system for investigating mechanisms of meiosis and oocyte maturation. We have used a proteomics approach to identify novel components involved in progesterone induced meiotic maturation in this system. Lysates from stage VI oocytes and mature eggs were labeled with CyDye DIGE Fluor minimal dyes and analyzed with 2-dimensional gel electrophoresis. A total of 51 proteins exhibiting statistically significant ($p<0.05$) changes were detected. Changes resulted from either altered level of expression or post-translational modification. Maximal change in expression was approximately 12 fold with the majority of proteins exhibiting decreased expression following maturation. Using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry we have to date identified a subset of these proteins. In this group are proteins known to play regualtory roles in the maturation process including elongation factor 1 gamma, a substrate of Cdc2 kinase. Subsequent to the identification of additional proteins we intend to select proteins of interest for further functional analysis. This will include microinjection of recombinant proteins, mRNA, antisense morpholinos and antibodies as well as experiments with specific inhibitors. During the course of this work we have also identified a change in the metabolism of the yolk storage proteins during oocyte maturation. Subsequent to germinal vesicle breakdown, the storage and metabolism of yolk proteins was found to be altered. The changes in yolk proteins during oocyte maturation are currently being investigated using techniques to measure changes in the pH of yolk storage platelets, electron microscopic examination of platelets, and immunofluorescence. We are attempting to integrate changes in vitellogenin metabolism within the framework of changes in signal transduction, metabolic, and cell division proteins identified through the proteomics analysis.

2316

**A Model for Bi-stability, Collapse, and Maintenance of Meiotic Spindles**

K. S. Burbank,[1] D. E. Fisher,[1] T. J. Mitchison[2]; [1]Physics, Harvard University, Cambridge, MA, [2]Systems Biology, Harvard Medical School, Boston, MA

We develop a simple model of the meiotic spindle with several novel components, analyze the model mathematically and with simulations, then test novel predictions with experiments. The components of our model are nucleation and sorting of microtubules near the chromosomes, microtubule sliding and bundling due to molecular motors, and loss of microtubules due to dynamic instability. Mathematical analysis shows that these components are capable of robustly forming a steady-state spindle of constant length. Our model recapitulates three important experimental results: When anti-parallel sliding is decreased, simulating Eg5 inhibition with monastrol, the system becomes bi-stable, with short "collapsed" and

long "bi-polar" solutions. When microtubule end bundling is sufficiently decreased, mimicking dynein inhibition, the spindle lengthens at the flux rate. Finally, when both bundling and anti-parallel sliding are inhibited in the model, the tendency towards collapse is lost. We test the model's prediction and experimentally find a previously unobserved dependence of spindle length on the speed of anti-parallel sliding, in good agreement with the model predictions.

2317

### The Drosophila lkb1 Gene is Required for Proper Centrosome Behavior

S. Bonaccorsi,[1] M. Giansanti,[1] B. Bolkam,[2] B. Williams,[2] V. Mottier,[1] M. L. Goldberg,[2] M. Gatti[1]; [1]Genetics and Molecular Biology, Università di Roma, Rome, Italy, [2]Molecular Biology and Genetics, Cornell University, Ithaca, NY

We have isolated lethal mutations in the lkb1 gene, the Drosophila homologue of C. elegans par-4 and human LKB1 mutated in Peutz-Jeghers syndrome. Flies homozygous for lkb1 lethal mutations die at the larval/pupal boundary, allowing cytological analysis of cell division in both mitotic and meiotic tissues. The spindles of lkb1 mutant neuroblasts display small asters and a low microtubule (MT) density, which do not appear to be due to a reduced MT nucleation ability of the centrosomes. As a result of the reduced stability of spindle MTs, lkb1 mutant brains also exhibit a metaphase-arrest phenotype and frequent polyploid cells. In addition, lkb1 mutations disrupt some of the asymmetries that characterize neuroblast divisions, including the different size of the centrosomes during ana-telophase and the positioning of the cleavage furrow. In contrast to mitotic spindles, meiotic spindles of lkb1 mutant males do not exhibit a reduction in microtubule density and/or stability. However, lkb1 spermatocytes are clearly defective in centrosome migration to the opposite cell poles. This is particularly evident in the second meiotic divisions, which often exhibit peculiar bipolar, monastral spindles with two centrosomes at the same pole. Collectively, these results indicate that Drosophila Lkb1 is required for different aspects of mitotic and meiotic cell division, suggesting that this kinase acts on multiple substrates required for proper spindle assembly and centrosome behaviour.

2318

### An RNAi Screen to Identify Genes Required for Meiotic Spindle Formation in *C elegans*

S. M. Wignall, A. Villeneuve; Developmental Biology, Stanford University, Stanford, CA

During cell division in most cell types, duplicated centrosomes nucleate microtubules and ultimately form the poles of the spindle. However, oocytes of many species lack centrosomes and therefore spindle assembly occurs through a different pathway. Our goal is to investigate centrosome-independent spindle formation by identifying proteins that promote spindle assembly and chromosome segregation in *C. elegans* oocyte meiosis. To this end, we have performed a targeted RNAi screen of genes that were previously classified as embryonic lethal in genome-wide screens. This class includes genes known to be required for acentriolar meiotic spindle assembly, such as mei-1 and mei-2 (regulators of microtubule stability) and klp-18 (a kinesin-like protein required for spindle morphogenesis). We visually screened the approximately 800 genes in this class for defects in spindle formation and/or chromosome alignment using worms co-expressing GFP::histone and GFP::tubulin to visualize chromosomes and microtubules simultaneously. As expected, we observed dramatic defects in meiotic spindle morphology following RNAi of mei-1, mei-2 and klp-18. In addition, we have identified a number of other genes whose inhibition alters the appearance of meiotic spindles. These include proteins uncharacterized in meiosis but known to play roles in mitosis, such as CLS-2 (a homolog of the microtubule-associated protein (MAP) CLASP), and the dynein-associated proteins NUD-1 and LIS-1. In addition, we have identified a number of genes uncharacterized in worms. Some of these have putative homologs in other organisms. For example, one is highly homologous to centractin, a component of the dynactin complex, and another shares homology with ASP, a *Drosophila* MAP. Finally, we identified one gene with no obvious homologs in other organisms. Future characterization of the genes identified in our screen may help us better understand the mechanisms involved in centrosome-independent spindle assembly.

2319

### Meiotic Segregation of Holocentric C elegans Chromosomes is Independent of the Centromeric Histone Variant CENP-A

J. W. Monen, P. Maddox, F. Hyndman, K. Oegema, A. Desai; Cellular and Molecular Medicine, Ludwig Institute for Cancer Research/UCSD, La Jolla, CA

Two distinct chromosome architectures are prevalent among eukaryotes: monocentric, in which localized centromeres restrict kinetochore assembly to a single chromosomal site, and holocentric, in which diffuse kinetochores form along the entire chromosome length. During mitosis, both chromosome types use specialized chromatin, containing the histone H3 variant CENP-A, to direct kinetochore assembly. For the segregation of recombined homologous chromosomes during meiosis, monocentricity is thought to be critical to limit spindle-based forces to one side of a crossover and prevent recombined chromatids from being simultaneously pulled towards both spindle poles. The mechanisms that allow holocentric chromosomes to avert this fate remain uncharacterized and are the subject of our investigation. We have found a dramatic difference in the mechanisms that segregate holocentric chromosomes during meiosis and mitosis in the nematode *Caenorhabditis elegans*. In late diakinesis, immediately prior to oocyte meiotic segregation, outer kinetochore proteins are recruited to cup-like structures on the chromosome surface via a mechanism independent of CENP-A. Formation of these structures occurs coincident with oocyte nuclear envelope breakdown; prior to this event localization of outer kinetochore proteins on chromosomes is CENP-A dependent. In striking contrast to mitosis, both oocyte meiotic divisions proceed normally following depletion of either CENP-A or the closely associated centromeric protein CENP-C. These findings highlight a dramatic difference between the segregation of holocentric chromosomes during meiosis and mitosis and demonstrate the potential to uncouple assembly of outer kinetochore proteins from CENP-A chromatin.

2320

### Maintenance of Chromatin Organization Between Meiotic Divisions in Surf Clam Oocytes

O. L. George, M. Johnston, C. B. Shuster; Biology, New Mexico State University, Las Cruces, NM

Meiotic divisions represent specialized cell cycles where cells undergo two reductive divisions without an intervening S phase. During oogenesis, the germinal vesicle breaks down, and following spindle assembly and migration to the cell cortex, the oocyte procedes into anaphase I and extrudes the first polar body. Between meiosis I and meiosis II, the paired sister chromatids remain condensed in contrast to mitotic divisions

where cytokinesis is always accompanied by chromatin decondensation and nuclear envelope reformation. Because other aspects of M phase exit are normal, we sought to dissect the mechanisms that drive cytokinesis while retaining chromatin condensation in Spisula solidissma oocytes. Oocytes were arrested in interkinesis following MI with the protein synthesis inhibitor emetine, and both histone H3 phosphorylation and spindle pole duplication occurred normally in comparison to controls. Neither inhibition of CDK1- nor MAPK activity in arrested oocytes was sufficient to drive chromatin decondensation or nuclear envelope reformation, suggesting that these kinases were not responsible for the maintenance of chromatin condensation. However, inhibition of Aurora B kinase activity resulted in chromatin decondensation, loss of histone H3 phosphorylation and reformation of the nuclear envelope. Inhibition of Aurora B activity during MI resulted in both a failure to extrude polar bodies and loss of histone H3 phosphorylation, two previously reported Aurora B-dependent processes. Together, these results suggest that extended Aurora B activity between meiotic divisions maintains chromatin condensation, thus allowing for the rapid reassembly of the MII spindle and progression through meiosis. Sponsored by U56CA096288.

2321

### Spindle Dynamics, Chromosome Elimination, and Non-disjunction in *Sciara* Male Meiosis

B. de Saint Phalle,[1,2] J. R. LaFountain,[3] R. Oldenbourg,[1] S. Inoue,[1] S. A. Gerbi[2]; [1]ADLC, MBL, Woods Hole, MA, [2]Biomed. Division, Brown University, Providence, RI, [3]Biological Sciences, University at Buffalo, Buffalo, NY

Spindle dynamics and chromosome segregation are unique in the fly, *Sciara*. Our studies utilize polarization microscopy for time lapse imaging of living *Sciara* spermatocytes. The new LC-PolScope is an orientation-independent liquid crystal polarizing microscope that allows quantitative examination of *Sciara* meiosis. A centrifuge polarizing microscope can isolate the meiosis I monopolar spindle. Immunostaining localizes regulatory proteins and motor proteins involved in spindle construction and chromosome motion. In spermatogenesis, the meiosis I spindle is monopolar. Chromosomes move from prophase to anaphase without congressing to a metaphase plate. The chromosomes are imprinted: maternally derived chromosomes and germ line limited chromosomes migrate to the single pole; paternally derived chromosomes move away from the single pole and are expelled in a bud. In meiosis II, a bipolar spindle forms, but it has a centrosome at only one pole. The chromosomes divide normally except for the X dyad, which remains at the pole with the centrosome. The nullo-X product of meiosis II is discarded in a bud. The metaphase checkpoint may not operate in meiosis I and may not apply to the X-dyad in meiosis II. The zygote has 3 X's. Chromosome elimination in an early cleavage division removes one or two paternally derived X's to determine the sex of the embryo. Translocations reveal that the cell recognizes the X dyad in meiosis II and in embryonic cleavages by a control element (CE) that is embedded within the tandem rDNA repeats which only functions in *cis*. In the embryonic cleavage division where X elimination occurs, the CE maintains cohesion of the chromosome arms in anaphase, while allowing separation of the X centromere on the sister chromatids. As a result, the X chromosomes remain on the metaphase plate and are eliminated.

## Cytokinesis II (2322-2339)

2322

### The Nocut Pathway links Abscission to Proper Function of the Spindle Midzone and Prevents Chromosome Breakage by the Cytokinetic Machinery

C. Norden, M. Mendoza, Y. Barral; Institute of Biochemistry, ETH-Zurich, Zurich, Switzerland

Tight coordinated of cytokinesis with chromosome segregation ensures that during cell division each daughter cell receives a full set of chromosomes. In yeast, the onset of actomyosin ring contraction and furrowing depends on activation of mitotic exit network and cyclin-kinase inactivation. Subsequently, abscission resolves the plasma membrane into two independent membranes. We know little about how this last and only irreversible step is controlled. Here, we show that a signalling pathway, which we call NoCut, represses abscission in cells with spindle midzone defects. Cells lacking a functional spindle midzone, such as *ase1*Δ and *ndc10-1* cells and mad2 cells treated with nocodazole, undergo proper actomyosin ring assembly and contraction but fail abscission. This defect depends neither on the lack of kinetochore-microtubule attachment nor on defects of cytoplasmic microtubules. Inactivation of aurora/Ipl1 and the suppressing the midzone assembly defects, indicating that the spindle midzone is not intrinsically required for cytokinesis. Epistasis analyses indicate that Ipl1 and anillins function in a signalling pathway, which we call NoCut, transducing an inhibitory signal from the spindle to the site of abscission. Ipl1 acted upstream of anillins and controlled their translocation from the nucleus to the bud neck in response to spindle defects. Inactivation of NoCut led to premature abscission and breakage of the chromosomes still engaged in the cleavage plane. NoCut inactivation was also lethal in cells with a fragile midzone. We propose that NoCut monitors spindle elongation and chromosome separation to prevent that cytokinesis is completed before the cleavage plane is cleared from chromatin.

2323

### Contractile Ring-Independent Localization of DdINCENP, a Protein Important for Spindle Stability and Cytokinesis

Q. Chen, A. De Lozanne; Institute of Cellular and Molecular Biology, and Department of MCDB, The University of Texas at Austin, Austin, TX

Chromosomal-passenger proteins display a very dynamic distribution during mitosis and cytokinesis and play important roles in cell division. Currently it is not know how chromosomal-passenger proteins localize at the cleavage furrow and what is their precise role in cytokinesis. To explore this question we analyzed the function of the *Dictyostelium* DdINCENP protein. Disruption of the single DdINCENP gene revealed important roles for this protein in mitosis and cytokinesis. DdINCENP null cells lack a robust spindle midzone and are hypersensitive to microtubule depolymerizing drugs suggesting that their spindles are unstable. Furthermore DdCP224, a protein homologous to the microtubule-stabilizing protein TOGp/XMAP215, was absent from the spindle midzone of DdINCENP null cells. Overexpression of DdCP224 rescued the weak spindle midzone defect of DdINCENP null cells. This suggests that DdCP224 is a possible downstream effector of the chromosome-passenger protein complex to stabilize the spindle midzone. In addition, we show that the localization of DdINCENP at the cleavage furrow is not dependent on the contractile ring but is modulated by myosin II. DdINCENP is normally found on the entire cortex region of the cleavage furrow during cytokinesis. In the absence of myosin II, DdINCENP is still localized at the cleavage furrow, but it forms a very narrow band at the equatorial plane of the dividing cell. Finally, analysis of cytokinesis in multinucleate cells demonstrated that while the contractile ring is assembled

at both normal equatorial and ectopic "Rappaport" furrows, DdINCENP only localized at the equatorial furrows. This suggests that the translocation of DdINCENP to the cleavage furrow at the end of mitosis occurs by a mechanism different from that controlling the formation of the contractile ring.

2324

**Regulation of Endosomal Membrane Trafficking is Required During Cytokinesis**

J. K. Schweitzer,[1] E. Burke,[1] H. Goodson,[2] C. D'Souza-Schorey[1]; [1]Biological Sciences, University of Notre Dame, Notre Dame, IN, [2]Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN

Membrane rearrangements are a vital part of cytokinesis. For example, proteins that mediate events such as membrane fusion and exocytosis have been shown to be essential for the successful completion of cytokinesis. In addition, our previous work has shown that ARF6, a key regulator of endosomal trafficking during interphase, is required for cytokinesis. Here we have examined endosomal trafficking during cytokinesis and assessed its importance for the successful completion of cytokinesis. Our results, using both live- and fixed-cell imaging, show that although membrane traffic is inhibited during the early stages of mitosis, endocytosis occurs during cleavage furrow ingression and cytokinesis in a regulated manner. During anaphase and early telophase, endocytic markers are internalized from the plasma membrane near the spindle poles, or polar regions, of dividing cells. However, during late telophase and cytokinesis, markers are actively endocytosed from the plasma membrane at the ingressed cleavage furrow, or midbody. Furthermore, we find that ligands endocytosed from the polar region of a dividing cell are trafficked to the midbody region as cytokinesis progresses. To determine whether or not receptor-mediated endocytosis is important for cytokinesis, we have examined mitotic cells transfected with AP180 or dominant negative Eps15 and found that they induce cytokinesis defects such as excessive membrane blebbing at the cleavage furrow and binucleated cell formation. We have also found that caveolae-mediated uptake may impact the completion of cytokinesis since caveolin-1 localizes to the midbody region during the late stages of cytokinesis and cholesterol depletion using cyclodextrin or filipin blocks the completion of cytokinesis. Taken together, our findings indicate that endosomal membrane trafficking is regulated during the late stages of mitosis and that it is important for the successful completion of cytokinesis.

2325

**The Double Life of the Substrate-Specific Adaptor MEL-26**

S. Luke-Glaser,[1] L. Pintard,[2] C. Lu,[3] P. E. Mains,[3] M. Peter[1]; [1]Biology, ETH Zurich, Zurich, Switzerland, [2]Samuel Lunenfeld Research Institute, Toronto, ON, Canada, [3]University of Calgary, Calgary, AB, Canada

BACKGROUND: The initiation of a cleavage furrow is essential to separate cells during cytokinesis, but little is known about the mechanisms controlling this actin-driven process. Previous studies in *C. elegans* embryos revealed that inactivation of the CUL3-based E3-ligase activator *rfl-1* results in an aberrant microtubule network, ectopic furrowing during pronuclear migration and defects during cytokinesis. RESULTS: Here we show that MEL-26, a substrate-specific adaptor of the CUL-3-based-E3-ligase, is required for efficient cell separation and cleavage furrow ingression during the *C. elegans* early mitotic divisions. Loss of MEL-26 function leads to delayed onset and slow ingression of cytokinesis furrows that frequently regress. Conversely, increased levels of MEL-26 in *cul-3(RNAi)* and *rfl-1* mutant embryos cause a hypercontractile cortex, with several simultaneously ingressing furrows during pronuclear migration. MEL-26 accumulates at cleavage furrows, and binds the actin-interacting protein POD-1. Importantly, POD-1 is not a substrate of the MEL-26/CUL-3 ligase, but is required to localize MEL-26 to the cortex. CONCLUSION: Our results suggest that MEL-26 not only acts as a substrate-specific adaptor within the MEL-26/CUL-3 complex, but also promotes cytokinesis by a CUL-3- and microtubule-independent mechanism.

2326

**Quantitative Analysis of Furrow Ingression during the First Mitotic Division of the *C elegans* Embryo**

L. K. Lewellyn, A. Audhya, A. Desai, K. Oegema; Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, La Jolla, CA

Cytokinesis partitions the cytoplasm and cell surface of a single cell to form two daughter cells following chromosome segregation in anaphase. During cytokinesis, a region of differentiated cortex called the contractile ring, which is enriched in actin and myosin II forms around cell equator. Constriction of the contractile ring changes the shape of the cell to facilitate cytokinesis. To study furrow ingression, we have developed a quantitative assay based on a *C. elegans* strain expressing a fusion of GFP with a PH domain that binds with high affinity to a phosphoinositide lipid ($PI4,5P_2$) that is specifically generated on the plasma membrane. In this method, z-series of the embryo are collected at 20s intervals and 3D reconstructions of the central region of the embryo allow us to monitor the kinetics of furrow ingression in an "end-on" manner. Using this assay even subtle defects in furrow ingression can be detected. In *C. elegans*, the first embryonic mitosis can be imaged following efficient RNA interference (RNAi)-mediated depletion of specific target proteins (under stringent conditions proteins are typically >95% depleted). We are using this capability to compare furrow ingression in control embryos to that in embryos depleted of essential proteins that have been implicated in cytokinesis.

2327

**PtdIns(4,5)P2 Functions at the Cleavage Furrow During Cytokinesis**

S. J. Field,[1,2] N. Madson,[2] M. L. Kerr,[2] K. A. A. Galbraith,[1,2] C. E. Kennedy,[2] M. Tahiliani,[2] A. Wilkins,[2] L. C. Cantley[2]; [1]Medicine, University of California San Diego, La Jolla, CA, [2]Divison of Signal Transduction and Department of Systems Biology, Beth Israel-Deaconess Medical Center and Harvard Medical School, Boston, MA

Phosphoinositides play important roles in the regulation of the cytoskeleton and vesicle trafficking, potentially important processes at the cleavage furrow. However, it remains unclear which, if any, of the phosphoinositides play a role during cytokinesis. Here we report a systematic, unbiased analysis of the role of the phosphoinositides during cytokinesis. We discover that PtdIns(4,5)P2 (but not other phosphoinositides) specifically accumulates at the cleavage furrow in every mammalian cell type examined. An enzyme that generates PtdIns(4,5)P2, PtdIns(4)P-5-kinase-α, similarly localizes to the cleavage furrow. Interference with PtdIns(4,5)P2 disrupts adhesion of the contractile ring to the plasma membrane. Furthermore, four distinct interventions to interfere with PtdIns(4,5)P2 each impair cytokinesis. We conclude that PtdIns(4,5)P2 accumulates at the cleavage furrow and is required for normal cytokinesis. At least one role for PtdIns(4,5)P2 is to function in adhesion of the plasma membrane to the

contractile ring, allowing the contractile ring to carry the plasma membrane inward to bisect the cell.

2328

**Positioning the Cell Division Plane in Fission Yeast: Mid1p, the Nucleus and the Endoplasmic Reticulum**

N. Padte,[1] R. R. Daga,[1] J. Bahler,[2] F. Chang[1]; [1]Microbiology, Columbia University College of Physicians and Surgeons, New York, NY, [2]Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom

In *Schizosaccharomyces pombe*, the position of the pre-mitotic nucleus specifies the cell division plane. Mid1p is a key protein in division site positioning, as *mid1* mutant cells form misplaced contractile rings and septa. Mid1p localizes to the nucleus and to a cortical band of dots overlying the nucleus during interphase. During mitosis, the broad band coalesces with other ring proteins to form the contractile ring. The positioning of the interphase mid1p cortical band is linked to nuclear position. For instance, the mid1p band moves with the nucleus as the nucleus re-centers after cell centrifugation. How mid1p senses the position of the nucleus remains elusive. We hypothesize there exists a physical linkage between the nucleus and the cell cortex which allows mid1p to constantly monitor nuclear position. One candidate for such a linkage is the endoplasmic reticulum (ER). Time-lapse images show that the ER forms dynamic threads between the nuclear envelope and the cell cortex region surrounding the nucleus. An overexpression screen for genes that produce a *mid1*-like phenotype identified a protein implicated in protein sorting and translocation at the ER. We are currently testing the role of the ER and this protein in cytokinesis and division site positioning.

2329

**Induction of Contractile Ring Assembly during Interphase by the Fission Yeast Formin Cdc12p**

A. Yonetani, F. Chang; Microbiology, Columbia University, New York, NY

Successful cytokinesis requires that the assembly and closure of the actomyosin contractile ring are properly regulated during the cell cycle. In *Schizosaccharomyces pombe*, the actin ring assembles in early mitosis and begins to contract in late anaphase. The formin cdc12p is a critical factor in ring assembly, as it nucleates actin filaments within the ring. We are interested in how the cdc12 formin is temporally and spatially controlled. We have found that expression of a truncated cdc12p lacking the C-terminal domain leads to deregulated actin ring assembly in wild type cells. We acquired time lapse movies of ectopic ring formation in cells co-expressing GFP-tubulin as an indicator of cell cycle state. Strikingly, cells expressing truncated cdc12p protein can assemble actin rings during interphase. Some ectopic rings appear to arise from remnants of previous rings, while others form *de novo* in the vicinity of the interphase nucleus. Further, some of these interphase actin rings can contract and even guide septation; while in other cells, rings wait to drive cleavage at the appropriate time in mitosis. Our observations suggest that the misregulation of cdc12p is sufficient to bypass cell cycle controls and to trigger not only F-actin assembly, but the entire process of cytokinesis.

2330

**Counting Cytoskeletal Proteins Molecules Globally and Locally in Fission Yeast**

J. Wu, T. D. Pollard; MCDB, Yale University, New Haven, CT

To understand the cytoskeletal dynamics in living cells one must know the global and local concentrations of the participating proteins. Concentrations indicate stoichiometries, determine rates of biochemical reactions, and are prerequisites for mathematical modeling. Unfortunately, concentrations are rarely available. We used spinning disk confocal microscopy to measure global and local concentrations of 28 cytoskeletal and signaling proteins fused to yellow fluorescent protein (YFP) in the fission yeast *Schizosaccharomyces pombe*. Native promoters controlled expression of these functional fusion proteins. Fluorescence measured by microscopy or flow cytometry was directly proportional to protein concentration measured by quantitative immunoblotting. Global cytoplasmic concentrations ranged from 40 (formin Cdc12p) to 63,000 nM (actin). The concentrations of most proteins are constant across the cell cycle and <50% of each protein is recruited to the division site during cytokinesis. By measuring local concentrations, we find each actin patch consists of ~200 50-nm long actin filaments, half of which are capped, and each crosslinked by 2-3 fimbrins. Fission yeast begin cytokinesis when anillin-like Mid1p exits the nucleus and initiates assembly of at least seven proteins in an equatorial broad band of ~75 dots. Each dot has ~21 molecules of Mid1p, 43 myosin-II Myo2p, 35 myosin regulatory light chain Rlc1p, 289 myosin essential light chain Cdc4p, 23 IQGAP Rng2p, 22 PCH protein Cdc15p, and 4 formin Cdc12p. Thus the stoichiometry of Mid1p to dimeric myosin-II to IQGAP to Cdc15 is close to 1:1:1:1. Formin homodimers nucleate and remain attached to the barbed end of elongating actin filaments. Thus each dot could grow two actin filaments anchored by Cdc12p, sufficient for myosin-II to pull the dots together into a continuous contractile ring early in mitosis. Our approach can be used to measure the global and local concentration of any fusion protein.

2331

**A Role for the Septation Initiation Network in the Formation of the Cytokinetic Ring in Fission Yeast?**

O. Hachet, V. Simanis; Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland

In most eukaryotes, cytokinesis is mediated by a contractile actomyosin ring (CAR). The formation of the CAR depends on the reorganization of the actin cytoskeleton at the onset of mitosis. In *Schizosaccharomyces pombe*, the onset of septation is temporally coordinated with the end of mitosis through the combined action of the septation initiation network (SIN) and the polo kinase Plo1p. The function of the SIN is to trigger the formation of the division septum and the contraction of the CAR after completion of chromosome partitioning. CAR assembly occurs at the onset of mitosis, independently of SIN signaling. However, ectopic activation of *spg1* is sufficient to trigger CAR assembly in interphase, though how this is achieved remains unclear. We are making use of *spg1* overexpression to evaluate the involvement of the SIN in CAR assembly.We characterized the properties of rings induced by *spg1* overexpression in G2 arrested cells. Using ring markers such as cdc15-GFP, rlc1-GFP and myp2-YFP, we found that interphasic *spg1* induced rings are functional in that they can contract, however some fail to be correctly positioned. This observation show that interphasic *spg1* induced CAR lack some spatial information suggesting a bypass of the CAR positioning mechanism or a failure to maintain CAR positioning. Using different genetic backgrounds, we addressed the requirements for the formation of interphasic *spg1* induced CAR. Our results suggest a requirement of the SIN pathway for the formation of such rings. These observations suggest that, although the SIN is not required for ring assembly, the SIN pathway may participate in this process.

2332

**Mutations in the *smoA* Gene Bypass the Requirement of Sin for Septation in *Aspergillus nidulans***

J. Kim, R. Shao, B. Liu; Section of Plant Biology, University of California-Davis, Davis, CA

Many filamentous ascomycetes like *Aspergillus nidulans* form mycelia with multinucleate cells. It is largely unknown how septation is temporally regulated in their mycelia. In *A. nidulans*, the AnMOB1 protein of the septation initiation network (SIN) is essential for septation and conidiation, but is not required for hyphal extension and colony formation. To isolate novel septation regulators, by UV mutagenesis we have isolated suppressor (*smo*) mutations that restored septation and conidiation when AnMOB1 was not expressed. Among more than 100 independent *smo* mutants, four recessive mutations at the *smoA* locus conferred identical phenotype of growth inhibition with wavy hyphae. The *smoA* mutations rendered hypersensitivity to low doses of the microtubule-depolymerizing agent benomyl. In *smoA* mutant cells, cytoplasmic microtubules became shorter than those of wild type. The *smoA* gene was cloned by DNA-mediated complementation, and it encodes a polypeptide of 652 amino acids. Genes encoding SMOA homologs were detected in genomes of other filamentous fungi like *Neurospora crassa*. A functional GFP (green fluorescent protein)-AnSMOA fusion protein localized to the nucleus indicating that AnSMOA was a nuclear protein. Loss-of-function *smo* mutations also suppressed a mutation of the *sepH* gene encoding the central kinase of the SIN in *A. nidulans*. We conclude that AnSMOA and other SMO proteins likely act as regulators antagonizing the SIN pathway in *A. nidulans*. In the absence of the AnSMOA protein, the fungus does not require the SIN for septation and conidiation. This work was supported by the National Science Foundation.

2333

**A Complex Containing the Sm-protein CAR-1 and the RNA Helicase CGH-1 is Required for Embryonic Cytokinesis in *C elegans***

A. Audhya,[1] F. Hyndman,[1] I. X. McLeod,[2] A. S. Maddox,[1] J. R. Yates,[2] A. Desai,[1] K. Oegema[1]; [1]Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, La Jolla, CA, [2]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

Cytokinesis completes cell division by partitioning the intracellular constituents of one cell to form two topologically distinct daughter cells. Here we describe the characterization of CAR-1, a predicted RNA binding protein whose depletion results in a specific defect in cytokinesis. Consistent with a role in RNA metabolism, CAR-1 localizes to germline-specific RNA-containing P-granules and co-purifies with the essential RNA helicase CGH-1, which controls its localization. The atypical Sm domain of CAR-1, which is predicted to mediate an association with RNA, is dispensable for CAR-1 localization but critical for its function. The failure of cytokinesis in CAR-1 depleted embryos likely results from a pronounced defect in the structure of the anaphase spindle, which normally interacts with the cortex to promote the completion of cytokinesis. In CAR-1 depleted embryos, inter-zonal microtubule bundles that recruit Aurora B kinase and the kinesin ZEN-4 fail to form. Depletion of CGH-1 results in sterility, but partially depleted worms produce embryos that exhibit a nearly identical phenotype to that resulting from depletion of CAR-1. Cumulatively, these results suggest that CAR-1 and CGH-1 function together to regulate RNAs important for anaphase spindle structure and point to a connection between RNA metabolism and cytokinesis.

2334

**A Functional Domain Interference Assay (FDIA) Reveals that Peptide Motifs of Mago Nashi Are Involved in Development in *Marsilea***

C. M. Van der Weele, S. M. Wolniak; Cell Biology and Molecular Genetics, University of Maryland-College Park, College Park, MD

Spermatogenesis in the water fern *Marsilea vestita* is initiated by placing dry microspores in water. The single cell in the microspore undergoes nine successive division cycles to produce 7 somatic cells and 32 spermatids. Thereafter, each spermatid differentiates to become a coiled, ciliated gamete. Mago nashi is a highly conserved protein that functions in the exon-exon junction complex for pre-mRNA splicing, as a component in nonsense-mediated mRNA decay, and as a component in transport/localization of mRNAs for axis formation. The protein consists of several beta-sheet and alpha-helix domains. The beta sheets are exposed on one side of the protein while the opposite face consists of alpha helical domains that interact with the Y14 protein to form a heterodimer. Previously, we found that *Mv*-mago and *Mv*-Y14 are important for the specification of spermatogenous and jacket cells in the gametophyte. RNAi treatments, using dsRNAs made from *Mv*-mago and *Mv*-Y14, induced cytokinesis defects with cells of random sizes and anomalous shapes. Transcripts encoding pre-mRNA processing proteins, and proteins important for gametogenesis failed to localize in spermatogenous initials; instead, both were present in all cells of the gametophyte. Five peptides corresponding to different parts of the mago nashi protein were added to microspores in a series of functional domain interference assays (FDIA) to assess interactions between native proteins and mago nashi *in vivo*. One peptide exerted no effect on development while another induced rapid death, even at low concentrations. With the remaining peptides, development was arrested at specific phases, with characteristic patterns of incomplete cytokinesis and abnormal cell shaping. The FDIA experiments show that different motifs in the same protein affect development at distinct stages. (Supported by NSF grant MCB 0234423 to SMW).

2335

**Filamentous Growth and Septation in *Ustilago maydis***

K. Snetselaar, S. Yerrum, M. McCann; Biology, Saint Joseph's University, Philadelphia, PA

The corn smut fungus *Ustilago maydis* is a dimorphic phytopathogenic basidiomycete. The haploid non-pathogenic form grows asexually by budding, while mating, cell fusion and filamentous growth are required for pathogenicity. At least three morphologically and behaviorally distinct filaments have been observed in *U. maydis*: mating filaments, infection filaments and the filaments that proliferate in planta. Mating and infection filaments both exhibit unipolar growth with nuclei arrested in G2. Filaments formed within the plant are difficult to study, but they are mitotically active and appear to have a bipolar growth pattern. Mutants lacking adenylate cyclase (*uac* mutants) are constitutively filamentous and are used as a model of filamentous growth in *U. maydis*, although they cannot undergo mating, cell fusion or pathogenic growth unless supplemented with cAMP. In this study, we compared features of growth and cytokinesis between these filaments and wild-type cells. Filaments formed by *uac* mutants had unconstricted septa, like wild-type mating and infection filaments, but unlike septa formed during budding or pseudohyphal growth. However, unlike wild-type mating and early infection filaments, filaments of *uac* cells had a bipolar growth pattern similar to that of budding cells. Fluorescence microscopy using strains containing a gfp-beta tubulin fusion showed that unlike in wild-type budding cells, mitotic spindles form in the center of unconstricted cell compartments in *uac* filaments, apparently at the site where the new septum will form. The membrane label FM4-64 and several labels for cell wall components showed that compartments in filaments formed by *uac* mutants are separated by a single septum that

differs in development from both the double septum formed in budding cells and the septum formed in unipolar infection filaments. Filaments formed by *uac* mutants may be more like filaments formed in planta than to other *U. maydis* filaments.

2336

### Role of Centrins in *Trypanosoma brucei* Cytokinesis

A. Selvapandiyan,[1] P. Kumar,[2] J. C. Morris,[3] C. C. Wang,[2] H. L. Nakhasi[1]; [1]Division of Emerging and Transfusion Transmitted Diseases, OBRR, Food and Drug Administration, Bethesda, MD, [2]Pharmaceutical Chemistry, UCSF, San Francisco, CA, [3]Genetics, Biochemistry and Life Science Studies, Clemson University, Clemson, SC

Centrins are calcium binding cytoskeletal proteins involved in duplication and segregation of centrosome/basal bodies in eukaryotes. Recently, we showed that centrin1 in *Leishmania donovani*, a causative agent of fatal visceral leishmaniasis disease in humans, is essential for basal body duplication and cytokinesis (Journal of Biological Chemistry 2004, 279:25703-25710). Similarly we wanted to define the function of centrins in other trypanosomatids. The genome of a trypanosomatid, *Trypanosoma brucei* (www.genedb.org) the parasite that causes the African sleeping sickness in humans, shows the presence of five different putative centrin genes. Using RNAi methodology, we generated individual centrin gene knockdown clones in the procyclic form of *T. brucei*. Knockdown of centrin 1, 2 and 3 but not 4 and 5 expression affected the growth of the parasite *in vitro*. Northern blot analysis on the growth affected cells showed specific reduction in the mRNA levels of respective centrins. Flow-cytometry analysis indicated that the cells were blocked at the G2/M cell cycle stage. Further, loss of centrins 1, 2 and 3 expression resulted in cells with multi-nuclei, having more than one kinetoplast and multi-flagella. These multinucleated cytokinesis arrested cells undergo a form of programmed cell death based on TUNEL assays. The mechanism by which centrins play a role in the cytokinesis in the procyclic form of *T. brucei* will be discussed.

2337

### Mechanism of Cytokinesis Initiation in *Trypanosoma brucei*

P. Kumar, C. C. Wang; Pharma. Chem., UCSF, San Francisco, CA

Polo-like kinase (Plk) typically plays multiple roles in both mitosis and cytokinesis in eukaryotes. We report that TbPlk, the only Plk homolog in the ancient eukaryote *Trypanosoma brucei*, controls cytokinesis but not mitosis. We used RNAi to knockdown TbPlk expression in the procyclic form of *T. brucei* and after the depletion of TbPlk, we observed apparently normal spindle formation, chromosome segregation and nuclear division, with accumulation of multiple nuclei, kinetoplasts, basal bodies and flagella, indicating that duplication and segregation of these organelles proceeded normally. But the cells failed to divide. TbPlk thus apparently does not play a role in either mitosis or kinetoplast segregation. Instead, it controls only the initiation of cytokinesis. The procyclic-form *T. brucei* is also known to continue cell division under mitotic arrest to produce anucleate cells known as the zoids. The fact that no zoids were detected in TbPlk-depleted cells further supports the notion that TbPlk is not involved in mitotic regulation but only cytokinesis initiation. The intracellular localization of TbPlk in the flagellum attachment zones which may constitute the cleavage furrow further supports the conclusion that TbPlk plays an important role in initiating cytokinesis. This is, to our knowledge, the first report of a polo-like kinase that is not associated with mitosis. Our finding provides a new tool for dissecting the role of Plks in initiating cytokinesis and regulating the cell cycle across the broad range of eukaryotes.

2338

### Characterization of Centrin an MTOC's Protein

Z. Sanoguet, M. Campbell, B. Pastrana; Chemistry, University of Puerto Rico, Mayaguez, Puerto Rico

Recombinant *Chlamydomonas* centrin was used as a model protein to understand the effects of phosphorylation on protein conformation. The purified protein was phosphorylated *in vitro* using protein kinase A (PKA). The extent of phosphorylation was determined by Matrix Assisted Laser Desorption Mass Spectrometry (MALDI-MS). The conformational changes and the hydrogen / deuterium (H/D) exchange dynamics at the molecular level were studied using Fourier Transformed Infrared spectroscopy (FT-IR) and two-dimensional correlation analysis. This protein was observed to have a thermal transition temperature (Tm) of 112°C with a pre-transition at 70 °C, using Differential Scanning Calorimetry (DSC). The FT-IR results suggest that changes in the backbone occur prior to any other change when thermally perturbed while for the H/D exchange loops were affected first. Discussion of the results is relevant in describing its biological function and for future studies with sfi 1, a target protein, essential in cell division.

2339

### Calomitric Properties of a Centrosome Associated Protein: Human Centrin 2

V. Meza-Venencia, B. Pastrana-Ríos; Chemistry, University of Puerto Rico, Mayaguez Campus, Mayaguez, PR

Human centrin 2 (Hcen 2) is an acidic centrosomal protein of 19,738 Da, that is comprised of four subdomains, called EF-hands, which represent the calcium-binding sites. Human centrin 2 gene locus is codified on chromosomes X and had been designated as *Hcetn2*. Hcen2 may play a role in centrosome duplication and segregation during mitosis. Transformed bacterial *E. coli* cells with a recombinant molecule containing the Hcetn2 gene were grown in either 2xYT broth or $^{13}C$ isotope enriched minimal media and induced to over-express the Hcen 2 and $^{13}C$-Hcen 2, respectively. These recombinant proteins were then purified by using a of hydrophobic affinity chromatography followed by several anion exchange chromatographic separations. Highly pure Human centrin 2 and $^{13}C$-homogenously labeled protein were obtained for biophysical studies. Differential scanning calorimetric (DSC) study of Hcen 2 showed that this is a very stable protein with a pre-transition temperature around 85 $^{o}C$ and a thermal transition temperature at 120 $^{o}C$.

## Actin Dynamics & Assembly III (2340-2358)

2340

### Molecular Analysis of Filopodia Assembly and Disassembly in Drosophila Cells

S. Rogers; Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Filopodia are specialized, finger-like cellular processes that allow cells to interact with their environment and to communicate with other cells. Best

understood in the context of cellular motility, filopodia detect and integrate guidance cues during migration to direct cells to their proper destination. Filamentous actin forms the core structural scaffolding of filopodia and it is the dynamics of the actin cytoskeleton that drive the cycles of protrusion and retraction that allow them to sample the extracellular space. I have developed a novel model system to study the processes of filopodia assembly and retraction using cultured Drosophila cells. These cells allow direct observation of actin dynamics using high-resolution light microscopy and are susceptible to RNAi-mediated gene inhibition. Preliminary studies indicate that filopodia assembly in this system is consistent with the Convergent Elongation Model that predicts that the balance between capping and anti-capping actin-binding proteins is a key determinant of the architecture of the actin network. I will present data about the function of the anti-capping protein, Enabled, in this system and how its differential regulation may lead to filopodia disassembly in response to extracellular signaling events.

2341

**Ena/VASP Proteins in Filopodia Formation**

D. A. Applewhite,[1] M. Barzik,[2] S. Kojima,[1] M. R. Mejillano,[1] T. M. Svitkina,[3] F. B. Gertler,[2] G. G. Borisy[1]; [1]Cell and Molecular Biology, Northwestern University, Chicago, IL, [2]Biology, Massachusetts Institute of Technology, Cambridge, MA, [3]Biology, University of Pennsylvania, Philadelphia, PA

Ena/VASP proteins control actin dynamics and are important mediators for lamellipodia and filopodia dynamics. (*Cell* (2004) **118**; 363-373; *Annu. Rev. Cell Dev. Biol.* (2003) **19**; 541-64). However, the detailed mechanism of how Ena/VASP proteins enhance filopodia is not known. We determined the domains necessary for filopodia formation and tip targeting using $MV^{D7}$ cell lines with stable expression of Ena/VASP mutants. A filopodia formation assay was developed based on siRNA depletion of capping protein (CP). The parental $MV^{D7}$ line, which is deficient in all three mammalian (Mena/VASP/EVL) homologues, failed to support intense filopodia formation after CP depletion. The EVH2 domain alone restored the ability of filopodia induction as efficiently as full-length VASP and Mena proteins. Further dissection revealed the G-actin binding, the F-actin binding and the coiled-coil motifs within EVH2 domain were necessary for filopodial induction. Interestingly, the EVH2 domain localized along the length of filopodia instead of at the tips as observed for the full-length protein, and the localization was resistant to detergent extraction. The EVH1 domain alone did not support efficient filopodia induction but did correctly localize at the tips of filopodia, and was rapidly removed by detergent. Full length VASP was retained at filopodial tips after detergent extraction, suggesting that VASP at filopodial tips is bound to the actin cytoskeleton through the EVH2 domain. Fluorescence recovery after photobleaching (FRAP) analysis showed that full length EGFP-VASP did not exchange rapidly at filopodial tips but that the EGFP-EVH2 domain exchanged relatively rapidly (within 1 minute) along the length of filopodia. Our data suggest a model in which the EVH1 domain is essential for targeting of Ena/VASP proteins at filopodial tips whereas the EVH2 domain dynamically associates with actin filaments and is required to support filopodia protrusion. Supported by NIH GM64346 (DAA) and NIH GM62431 (GGB).

2342

**Bone Resorption by Osteoclasts Requires Cortactin-based Actin Assembly**

S. Tehrani,[1] R. Faccio,[2] F. P. Ross,[3] J. A. Cooper[1]; [1]Cell Biology, Washington University School of Medicine, Saint Louis, MO, [2]Orthopaedic Surgery, Washington University School of Medicine, Saint Louis, MO, [3]Pathology and Immunology, Washington University School of Medicine, Saint Louis, MO

Osteoclasts are essential for bone dynamics and calcium homeostasis. They form a tight seal on the bone surface, onto which they secrete acid and proteases to resorb bone. The sealing ring contains actin filaments, as do podosomes, which form when osteoclasts are plated on non-osteoid surfaces. Cortactin, a c-Src substrate known to promote Arp2/3-mediated actin assembly in vitro, is expressed in osteoclasts and localizes to the sealing ring and podosomes. To address the role of cortactin and actin assembly in osteoclasts, we depleted cortactin by RNA interference. Cortactin-depleted osteoclasts displayed a complete loss of bone resorption with no formation of sealing zones or podosomes. The cells were remarkably normal in other respects, including differentiation markers and actin-based motility of the cell edge. We found that cortactin localized strongly to the nascent podosome along with dynamin2, early in actin assembly. N-WASp, WASp and dynamin2 localization to the cortical actin cytoskeleton was disrupted in cortactin-depleted osteoclasts. Thus, cortactin has a critical and highly specific role in osteoclast function related to actin-based assembly of podosomes and sealing zones for bone resorption, and cortactin-based actin filament assembly at the sealing zone is necessary for bone resorption.

2343

**Yeast Actin Patches as a Model of the Actin Assembly at the Leading Edge**

V. A. Sirotkin,[1] K. Macmillan,[1] S. Yuan,[2] D. R. Kovar,[1] T. D. Pollard[1]; [1]MCDB, Yale University, New Haven, CT, [2]MBB, Yale University, New Haven, CT

Yeast actin patch assembly depends on Arp2/3 complex and shares many proteins with the leading edge of motile cells, including capping protein, profilin, actin cross-linkers, and cofilin. We used live cell imaging of fluorescent protein-tagged patch proteins to determine the effects of varying concentrations of patch components on patch dynamics. Fission yeast actin patches assemble at endocytic sites since patch assembly starts with recruitment of Pan1p and End4p, known to be involved in endocytosis. Downstream two parallel partially redundant pathways recruit Arp2/3 complex, one using WASp Wsp1p and another using myosin-I Myo1p. Patch dynamics are abnormal in the absence of either Myo1p or Wsp1p. Arp2/3 complex dissociates from these activators and moves centripetally. Coronin Crn1p joins the patch at the onset of mobile phase and remains in the patch longer than Arp2/3 complex. Actin capping protein, fimbrin and App1p accumulate and disperse in parallel with Arp2/3 complex throughout lifetime of the patch, indicating that actin filament capping and crosslinking happen within 1 s of filament nucleation by Arp2/3 complex. This is consistent with a network of short cross-linked filaments. In the absence of capping protein patch dynamics appear normal whereas capping protein over expression slows patch assembly, disassembly, and motility. Reduction in the level of profilin causes cytokinesis defect and depolarizes patches, but with only minor defects in patch dynamics. Elevated profilin concentrations delay Arp2/3 complex recruitment and slow patch motility. The observed changes in patch dynamics may be due to effects of capping protein and profilin concentrations on actin assembly at both actin patches and cables, known to associate with patches. The mechanism of actin assembly in patches appears similar to that at the leading edge of motile cells.

2344

**A Genetic Interaction Map for Actin Binding Proteins and Actin Surfaces in *S Cerevisiae***

J. A. Hendries; Brandeis University, Waltham, MA

Cellular actin networks are all assembled from the same basic building blocks, globular actin subunits, yet there is incredible diversity within actin filament arrays. This diversity is achieved through the combined activities of multiple actin binding proteins (ABPs) working in concert. To understand better how the actions of so many ABPs are coordinated *in vivo*, we performed a genetic screen in *S. cerevisiae* to define surfaces on actin that interact functionally with different ABPs. We tested all possible uni-directional and bi-directional suppression interactions between 23 non-lethal *act1* alleles and 17 different ABPs overexpressed on plasmids. 50 suppression interactions were detected. Some confirm previously identified functional links between specific actin surfaces and ABPs, but most represent new interactions. In addition, a comparison of genetic interaction profiles for individual ABPs with *act1* alleles suggested new and unexpected *in vivo* roles for certain ABPs. We selected a subset of these interactions for further *in vivo* analyses. Together, these data: (1) clarify how tropomyosin (Tpm1) associates with F-actin and the functional role of this interaction *in vivo*; (2) refine our understanding of how cofilin (Cof1) interacts with F-actin; and (3) reinforce the functional relationships among Aip1, Twf1 and Cof1 in promoting actin turnover. These data lend new insights into the mechanisms by which cells regulate actin dynamics and provide important tools for future genetic and biochemical investigations in this area. (240 words) Jacqueline A. Hendries, Meghal Gandhi, and Bruce L. Goode

2345

**Reconstitution of Actin Assembly in Cell-free Extracts and Development of a Functional Map of Actin Cytoskeleton Components in *S Cerevisiae***

J. L. DAgostino, A. L. Goodman, E. Smith, B. L. Goode; Biology, Brandeis University, Waltham, MA

Dynamic behavior of the actin cytoskeleton is regulated by the combined actions of a multitude of proteins having diverse activities on actin and each other. To understand the complete inner-workings of such a complicated biological machine will ultimately require obtaining a map of all of the components and their activities, cellular levels, and interactions. Toward this goal, we have developed a genetically and biochemically tractable assay for reconstituting actin assembly in *S. cerevisiae* cell-free extracts. Nucleation of actin assembly in this assay is dependent on Arp2/3 complex and WASp, but not formins or tropomyosin. The components of the actin structures assembled in extracts were defined by tandem mass spectrometry. They include most actin-associated proteins (AAPs) that reside on mature actin patches *in vivo*. In contrast, the signature component of actin cables, tropomyosin, was absent despite its abundance in extracts. Thus, the cell-free assay mirrors *in vivo* requirements for formation of actin patches, but not cables. In addition, we defined the cellular concentrations of actin and 13 AAPs and examined their physical interactions in stable complexes. We present a working map of the components of yeast actin binding proteins and their *in vivo* levels and interactions. This work represents an important step towards achieving a complete functional map of the actin cytoskeleton in a single living cell.

2346

**Srv2/CAP: The Grand Central Station of Actin Turnover**

O. Quintero-Monzon, B. L. Goode; Biochemistry, Brandeis University, Waltham, MA

Many cellular processes depend on the rapid remodeling of actin structures, including endocytosis, cytokinesis, and cell motility. This requires cells to rapidly disassemble older actin filaments and convert ADP-actin monomers to an ATP-bound assembly-competent state. ADF/coflilin and profilin have established roles in promoting actin turnover, but do not operate alone. Recent studies demonstrate that the highly conserved actin binding protein Srv2/CAP (cyclase-associated protein) plays a critical role in accelerating actin turnover in both yeast and animal cells. Previously, we isolated native yeast Srv2 in a 600kDa complex comprised entirely of Srv2 (53kDa) and actin (43kDa) in a 1:1 molar ratio, suggestive of a hetero-dodecameric structure. Further, our biochemical analyses showed that Srv2 accelerates cofilin-dependent actin turnover by recycling cofilin from ADP-actin monomers and that Srv2 functions with profilin to promote nucleotide exchange on G-actin. Here, we further dissect the Srv2 complex structure and its *in vivo* mechanism of promoting actin turnover. First, we have reconstituted active 600kDa Srv2 complex from recombinant full-length Srv2 expressed in *E. coli* and purified rabbit skeletal muscle actin. Second, we show that Srv2 alone forms a hexamer maintained by interactions of its N-terminal 50 amino acids. Disruption of this domain in vitro leads to dimeric Srv2, and in vivo this causes dramatic changes in cell morphology and actin organization. Thus, Srv2/CAP cellular function requires proper multimerization. Third, we have used specific point mutations to uncouple Srv2 interactions with four ligands (Abp1, profilin, cofilin, and G-actin) and dissect their combined mechanism of promoting actin turnover. Together, our data show that Srv2 forms a Mega-Dalton sized hub that coordinates dynamic interactions with actin and other factors to accelerate actin turnover *in vivo*.

2347

**Contractile Structures Formed during Oocyte Wound Healing Contain Discrete Sub Domains of Actin Dynamics**

B. Burkel, W. Bement; Zoology, University of Wisconsin, Madison, WI

F-actin plays an integral role in both cytokinesis and the closure of cortical wounds. During these events, the cell assembles a dynamic contractile ring of F-actin and myosin 2. While the importance of F-actin in these contractile structures has been established for some time, accurately assessing its temporal and spatial dynamics has been hindered in part by the shortcomings in available probes for imaging F-actin *in vivo*. These shortcomings range from perturbation of filament dynamics to the inability to distinguish F-actin from total actin populations. Here we have characterized a fluorescent fusion protein comprised of the first 261 amino acids of the actin binding protein, utrophin, that overcomes these shortcomings. This fusion protein, $Utr_{1-261}$, accurately reports F-actin populations, but does not alter actin dynamics or impact myosin 2-based contractility. By using this probe in conjunction with various photoactivation techniques, we have been able to establish that these contractile structures are divided into discrete sub domains that differ sharply with respect to their dynamics.

2348

**Involvement of Sterols and Phospholipid Asymmetry in Polarized Organization of the Actin Cytoskeleton Through Endocytic Recycling in Yeast**

T. Kishimoto, T. Yamamoto, K. Tanaka; Division of Molecular Interaction, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

Specific changes in membrane lipid composition are implicated in actin cytoskeletal organization, vesicle formation, and control of cell polarity. Most cell types display an asymmetric distribution of phospholipids across the plasma membrane that seems to be regulated by proteins mediating the transbilayer movement of phospholipids. We have previously reported that Cdc50p, a membrane protein in the endosomal/TGN compartments, controls polarized growth in yeast, and that Cdc50p is a non-catalytic subunit of the P-type ATPase Drs2p, which is proposed to translocate phospholipids across the TGN lipid bilayers. In this work, we show that the *cdc50Δ* mutation is synthetically lethal with mutations affecting the late steps of ergosterol synthesis (*erg2* to *erg6*). Defects in cell polarity and actin organization were observed in the *cdc50Δ erg3Δ* mutant. In particular, actin patches, which are normally found at cortical sites, were assembled intracellularly along with their assembly factors, including Las17p, Abp1p, and Sla2p. The exocytic SNARE Snc1p, which is recycled by an endocytic route, was also intracellularly accumulated, and inhibition of endocytic internalization suppressed the cytoplasmic accumulation of both Las17p and Snc1p. Confocal microscopy suggested that the intracellular actin patches were formed on the surface of unrecycled endocytosed membranes that contain Snc1p. These membranes overlapped with filipin-positive sterol-rich structures, suggesting that sterols accumulate in those membranes in the *cdc50Δ erg3Δ* mutant. In conclusion, simultaneous loss of phospholipid asymmetry and sterol structural integrity could lead to a defect in endocytic recycling, bringing about the assembly of cytoplasmic actin patches onto endocytic membranes that may be similar to the plasma membrane in terms of lipid composition.

2349

**Yeast as a Model for Retrograde Flow: Actin Assembly and Tropomyosin-Regulated Type II Myosin Drive the Retrograde Movement of Actin Cables**

T. M. Huckaba, T. G. Lipkin, L. A. Pon; Program in Cell Biology and Pathobiology, Columbia University, New York, NY

Actin cables in budding yeast are bundles that align along the mother-bud axis and serve as tracks for anterograde and retrograde organelle and particle transport. Actin cables undergo retrograde flow from their site of assembly in the bud and bud neck toward the mother cell tip at a rate of 3-4 microns/second. Previously, we showed that the retrograde flow of actin cables depends on actin assembly. Here, we show that the type II myosin, Myo1p, also contributes to actin cable retrograde flow. Deletion of the *MYO1* gene, delocalization of Myo1p from the bud neck, or a conditional mutation in the actin-binding site in the Myo1p motor domain all significantly decrease the rate of actin cable retrograde flow. Conversely, deletion of the *TPM2* gene, which encodes one of the two yeast tropomyosins, significantly increases the rate of actin cable retrograde flow. These findings raise the possibility that Tpm2p regulates Myo1p function in actin cable movement. Consistent with this, 1) yeast carrying mutations in *TPM2* and *MYO1* exhibit the same phenotype as *MYO1* mutation alone, and 2) purified Tpm2p but not Tpm1p inhibits Myo1p-driven microfilament gliding. These data support a model whereby bud neck-localized Myo1p drives actin cable movement, and Tpm2p regulates access of Myo1p to actin cables. Our study reveals novel functions for type II myosin and a tropomyosin isoform in budding yeast. Moreover, we find that retrograde actin cable flow in yeast resembles retrograde actin movement in the leading edge of motile cells: both processes are regulated by a tropomyosin isoform and driven by actin assembly and type II myosin. This suggests that actin retrograde flow occurs by a conserved mechanism that can be modeled in budding yeast.

2350

**Actomyosin Cortex Breaks and Drives Cell Shape Oscillations**

E. Paluch,[1] M. Piel,[2] J. Prost,[1] M. Bornens,[2] C. Sykes[1]; [1]UMR 168, Institut Curie / CNRS, Paris, France, [2]UMR 144, Institut Curie / CNRS, Paris, France

We investigate the spontaneous behaviors of the actomyosin cell cortex when the control of its contractility by microtubules is suppressed. Under these conditions and in the absence of substrate adhesions, the cell cortex spontaneously breaks and a membrane bulge devoid of detectable actin and myosin is expelled through the hole. A constriction ring at the base of the bulge then oscillates from one side of the cell to the other. The movement is accompanied by sequential redistribution of actin and myosin to the membrane. We observe this oscillatory behavior also in cell fragments of various sizes, providing a simplified, nucleus-free system for biophysical studies (1). We conclude that it reveals an intrinsic behavior of the actomyosin cortex. Our observations suggest a mechanism based on active gel dynamics and inspired by our study of symmetry breaking in actin gels growing around beads (2). The proposed mechanism for breakage of the actomyosin cortex may be used for cell locomotion and division. (1) Paluch E, Piel M, Prost J, Bornens M, Sykes C. 2005. Cortical actomyosin breakage triggers shape oscillations in cells and cell fragments. *Biophys. J.* **89**:724-733. (2) van der Gucht J, Paluch E, Plastino J, Sykes C. 2005. Stress release drives symmetry breaking for actin-based movement. *Proc. Natl. Acad. Sci. USA* **102**:7847-7852.

2351

**Actin Dynamics Play an Essential Role in Hemoglobin Uptake and Transport by *Plasmodium falciparum***

M. D. Lazarus, T. Schneider, T. F. Taraschi; Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, PA

Research indicates that host cell hemoglobin (hgb) degradation by intraerythrocytic *Plasmodium falciparum* (*Pf*) malaria parasites is essential for parasite viability. Mechanisms of Hgb uptake and transport by these intraerythrocytic parasites, however, are poorly understood. Hgb internalization begins through a double-membrane invagination located on the parasite's surface membranes, known as the cytostome (CYT). The CYT is internalized, forming a double membrane vesicle which is transported through the parasite cytosol and fuses with the lysosome-like organelle, the food vacuole (FV). Within the FV, cysteine proteases help break down the host-derived Hgb. Treatment of intraerythrocytic *Pf* with the actin filament-stabilizing drug Jasplakinolide (JAS) indicates that actin dynamics play an essential role in the parasite's Hgb uptake and transport pathway. Long-term JAS treatment arrests *Pf* development. These JAS-treated parasites lack internal organelles, as indicated by electron microscopy (EM) and their *Pf*LDH (an intraerythrocytic parasite development marker) is markedly reduced as compared to untreated parasites. *Pf*LDH assays indicate that short-term JAS treatment alters late-stage *Pf* development but does not affect early-stage parasites. Ultrastrucutural characterization of short-term JAS treated parasites reveals multiple CYT as well as unusually large, deranged CYT at the surface of the parasite

compartment. Treatment of intraerythrocytic *Pf* with the cysteine protease inhibitor, E-64, results in enlarged FV containing undegraded Hgb. Simultaneous treatment of *Pf* with JAS and E-64 results in a loss of effect of E-64 on the FV presumably due to the inability of the parasite to uptake and/or transport Hgb to the FV. Taken together, this data indicates that actin dynamics play an essential role in the uptake and transport of RBC Hgb to the FV and that these processes are essential for parasite development.

2352

**Diacylglycerol Kinase-ζ induces Membrane Blebbing in Myotubes via a Rho Kinase-Dependent Pathway**

J. C. Maillet,[1,2] R. Parks,[1,3] M. K. Topham,[4,5] S. H. Gee[1,2]; [1]Center for Neuromuscular Disease, University of Ottawa, Ottawa, ON, Canada, [2]Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, [3]Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada, [4]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, [5]Department of Internal Medicine, University of Utah, Salt Lake City, UT

Membrane blebbing is a dynamic event associated with cellular processes such as apoptosis and cell migration. The formation of membrane blebs requires the rearrangement of the actin cytoskeleton, which is mediated in part by the Rho GTPases. We previously showed that diacylglycerol kinase-ζ (DGK-ζ), which phosphorylates diacylglycerol to yield phosphatidic acid, associates with the Rho GTPase Rac1 in areas of C2C12 myoblasts undergoing actin cytoskeletal rearrangements. In muscle, DGK-ζ localization is regulated by its association with syntrophins, a family of scaffolding proteins belonging to the dystrophin glycoprotein complex. Here, we used immunofluorescence microscopy to study the effect of DGK-ζ on the actin cytoskeleton of differentiated C2 myotubes. Adenoviral-mediated overexpression of DGK-ζ induced membrane blebbing in myotubes but not myoblasts. By overexpressing various mutants it was determined that membrane blebbing was not dependent on the kinase activity of DGK-ζ. However, a mutant that does not interact with syntrophins failed to induce membrane blebbing. Furthermore, using inhibitor studies we show that membrane blebbing is dependent on the activity of Rho kinase (ROCK), a downstream effector of RhoA known to induce membrane blebbing via increased cell contractility. Lastly, using GST pulldowns we show that DGK-ζ associates with RhoA. Taken together these results suggest that syntrophins are required for DGK-ζ and RhoA to activate ROCK, resulting in membrane blebbing in myotubes.

2353

**A Role for Tropomodulin 3 in Regulation of F-actin Abundance and Organization in Polarized Epithelial Cells**

K. L. Weber, R. B. Nowak, V. M. Fowler; Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

The intestinal epithelium is composed of highly differentiated polarized epithelial cells, the function of which is dependent on the maintenance of cell polarity. Microvilli, which cover the apical surface of polarized epithelial cells thus imparting an essential increase in cellular surface area, consist of parallel bundles of actin filaments oriented such that the barbed-ends of the filaments are located at the microvillar tip, and the pointed-ends are located in the terminal web in the apical portion of the cell body. Despite the fact that microvillar actin filaments are dynamic, implying active regulation of length, very little data exists regarding binding partners for either their pointed or barbed ends. Caco-2 is an immortalized epithelial cell line derived from a human colon adenocarcinoma that faithfully represents microvilli-forming epithelial cells when cultured *in vitro*. Tropomodulin (Tmod) is an actin filament pointed-end capping protein. We have shown that Tmod3 localizes to the apical surface of intestinal crypt cells *in situ*, and is the sole Tmod isoform present in Caco-2 cells. Immunofluorescence staining of endogenous Tmod3 and infection with adenovirus expressing GFP-Tmod3 reveal that Tmod3 is localized to the apical region of the cell in the terminal web at the base of microvilli, colocalizing with fodrin, a terminal web marker. Tmod3 is also localized to the lateral membranes at cell-cell contacts where it partially colocalizes with E-cadherin. Tmod3 does not colocalize with actin in stress fibers at the basal surface of the cell. Interestingly and unexpectedly, we also saw occasional localization of Tmod3 at the tips of microvilli, where actin barbed-ends are concentrated. Preliminary siRNA experiments suggest a role for Tmod3 in regulating F-actin levels and organization at the apical surface and cell-cell junctions of polarized epithelia.

2354

**VEGF-induced LIM Kinase 1 Phosphorylation and Activation are Required for Actin Cytoskeletal Reorganization and Migration of Endothelial Cell**

M. Kobayashi, M. Nishita, K. Ohashi, K. Mizuno; Graduate School of Life Sciences, Tohoku University, Sendai, Japan

Cofilin plays an essential role for actin reorganization in motile cells. Cofilin is inactived by phosphorylation at Ser3 by LIM kinase (LIMK). Vascular endothelial growth factor (VEGF), which is a major regulator of angiogenesis, induces dynamically change in actin cytoskeletal reorganization and morphology in vascular endothelial cells. LIMK1 is activated through phosphorylation at Thr-508 by ROCK and PAK, which are downstream effectors of Rho and Rac/Cdc42, respectively. In the present study, we investigated the role of LIMK1 in VEGF-induced morphological changes in vascular endothelial cells (HUVECs and MSS31 cells). When the P-cofilin level was estimated by using two-dimensional gel immunoblot analysis with an anti-cofilin antibody, the ratio of P-cofilin increased to nearly equal amount to the unphosphorylated cofilin at 15 min after VEGF stimulation. We obtained evidence that VEGF significantly induces the activation of LIMK1 and cofilin phosphorylation through activation of p38 MAP-kinase and MAPKAP kinase-2 (MK2). Activated p38 and MK2 phosphorylated LIMK1 in cultured cells and in cell-free system at sites other than Thr-508. Activation of p38-MK2 pathway, induced by overexpression of constitutively active MKK6(DE), was sufficient for LIMK1 activation and stress fiber formation. Furthermore, a LIMK1-mutant, which is not phosphorylated by p38 and MK2, blocked VEGF-induced stress fiber formation and suppressed cell migration. These findings suggest that p38-MK2-pathway mediated LIMK1-activation significantly contributes to the VEGF-induced morphological changes and migration of vascular endothelial cells.

2355

**Intravital Visualization of Ischemia Induced Actin Alterations with 2-Photon Microscopy**

S. L. Ashworth,[1] G. A. Tanner,[2] R. M. Sandoval,[1] S. B. Campos,[1] J. R. Bamburg,[3] B. A. Molitoris[1]; [1]Medicine, Indiana University School of Medicine, Indianapolis, IN, [2]Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN, [3]Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO

Proximal tubule cell (PTC) microvilli microfilament destruction in response to renal ischemia leads to cellular and organ dysfunction. The precise dynamics of the actin cytoskeletal changes during ischemia and reperfusion in a live animal are unknown, but fixed tissue and cell culture studies

support the role of F-actin destruction in microvilli breakdown. To quantitatively analyze the effects of ischemia and reperfusion on the actin cytoskeleton in PTCs, we utilized adenoviral-mediated delivery of a fluorescently-tagged actin cDNA into the live animal and observed actin cytoskeletal changes using intravital 2-photon microscopy. Image stacks were collected of the actin-GFP expressing PTCs under control conditions, during 25 minutes of ischemia induced by clamping the suprarenal aorta, and at 8, 25, and 55 minutes of blood reflow. Metamorph, Voxx and Amira reconstruction softwares were used to quantitatively analyze actin alterations and cell volume changes during ischemia and recovery. Actin-GFP was expressed in PTCs and incorporated into apical microvilli microfilaments and in basal-lateral and cortical actin structures. With induction of ischemia, rapid breakdown of apical microvilli microfilaments occurred. Actin-GFP containing intraluminal vesicles or blebs were observed within ten minutes of ischemia induction. With blood reflow, the actin-GFP-filled blebs flowed down the tubule lumen. A 48% increase in PTC volume occurred during ischemia, which partially recovered to 26% over baseline during reperfusion. These live animal studies demonstrate the rapid dynamic nature and biological importance of F-actin apical microvilli breakdown and bleb formation as a consequence of renal ischemia.

2356

**Tropomyosins Regulate the Response of Actin Filaments to Cytochalasin and Latrunculin**

P. Gunning,[1,2] S. Creed,[1,2] N. Bryce,[1] R. Weinberger,[1] J. Stehn[1]; [1]Oncology Research Unit, The Children's Hospital at Westmead, Westmead NSW, Australia, [2]Discipline of Paediatrics and Child Health, University of Sydney, Sydney, Australia

Tropomyosins form a head-to-tail polymer which lies in the major groove of actin filaments. Over 40 isoforms of tropomyosin have been identified. Gene knock-out studies have shown that tropomyosins perform essential functions in a variety of organisms and that at least some isoforms are not functionally interchangeable. Isoform specific function appears to derive from the intracellular sorting of isoforms to different actin filament populations. In addition, isoforms differ in their ability to regulate actin-myosin and actin-cofilin interactions. In this study, we have addressed the ability of tropomyosins to regulate the response of actin filaments to the drugs cytochalasin and latrunculin in an isoform specific manner. Neuroepithelial cells which over-express specific tropomyosins were exposed to the drugs and actin filament response was measured by visualisation of actin organisation and partitioning of actin between Triton X-100 soluble and insoluble fractions. Tm5NM-1 induces stress-fibre formation whereas Tm3 induces small filipodia. Over-expression of Tm5NM-1 increases and Tm3 decreases partitioning of actin into the insoluble fraction relative to controls. Similarly over-expression of Tm5NM-1 increases the resistance of actin filaments to cytochalasin D and Latrunculin A as judged by Triton X-100 solubility and actin organisation. In contrast, Tm3 increases actin filament sensitivity to these drugs. The results suggest that Tm5NM-1 containing filaments exhibit slower turnover kinetics and are less susceptible to actin severing proteins than Tm3 containing filaments and that the Tms can directly regulate these processes.

2357

**Translocation of Rho Gtpase Activity Zones Occurs via Cortical Flow and Signal Treadmilling**

H. A. Benink, W. M. Bement; Zoology, University of Wisconsin - Madison, Madison, WI

Single-cell wound healing depends on the closure of a dynamic array of actin and myosin-2. Responsible for the formation and closure of this array are segregated RhoA and Cdc42 activity zones that form rapidly upon wounding. Once established, these GTPase activity zones maintain their segregation and translocate in concert with the actin array as the wound closes. To elucidate the means that control zone translocation we have used live 4D confocal imaging of Xenopus oocytes, chemical inhibitors of flow, photoactivatable GTPase activity probes and Fluorescence Recovery After Photobleaching (FRAP). Our results reveal that both cortical flow and signal treadmilling (the result of localized GTPase activation / inactivation within the observed zones of activity) are responsible for GTPase activity zone translocation.

2358

**Analysis of MIM and ABBA1, Two Homologous Regulators of the Actin Cytoskeleton**

P. K. Mattila,[1] J. Saarikangas,[1] M. Varjosalo,[2] J. Taipale,[2] M. Salminen,[1] P. Lappalainen[1]; [1]University of Helsinki, Institute of Biotechnology, Helsinki, Finland, [2]University of Helsinki, Molecular/Cancer Biology Program, Helsinki, Finland

The diverse essential functions of the actin cytoskeleton are regulated by a large number of actin-binding proteins. Two novel regulators of the actin cytoskeleton, MIM (missing in metastasis) and ABBA1, were recently identified from mammals. They are approximately 55 % identical to each other and are composed of an N-terminal actin filament bundling IMD domain and a C-terminal actin monomer-binding WH2 domain. Interestingly, MIM was recently also identified as an enhancer of Gli-dependent transcription of the Shh signalling pathway (Callahan et al., 2004). Our Northern blot and in situ hybridization analyses revealed that MIM and ABBA1 show distinct expression patterns. In adult mouse MIM is especially strongly expressed in liver, kidney and Purkinje cells, whereas ABBA1 is mainly expressed in spine and molecular layer of the cerebellum. Both proteins are also strongly expressed during the development. Over-expression of MIM and ABBA1 in various cell types resulted in the loss of stress fibers and appearance of abnormal actin structures and microspikes. To reveal the biological roles of these proteins, we have generated MIM knockout mice and are currently generating ABBA1 knockout mice. Mice lacking MIM are viable and do not display any gross abnormalities. The lack of obvious developmental defects in MIM -/- mice suggests that this protein does not play a central role in the Shh pathway during embryogenesis. To further investigate the role of MIM in Shh signalling, we carried out an in vitro Gli-lusiferace reporter assay in NIH-3T3 cells. Expression of MIM and ABBA1 had no effect on Hh signalling or on transcriptional activity with Gli1/2 in these cells. Together, these studies suggest that MIM and ABBA1 regulate certain specialized actin-dependent processes in mammals but do not significantly contribute to Shh signalling as previously suggested.

## Muscle: Biochemistry & Cell Biology II (2359-2375)

2359

**Aberrant Cytoarchitecture Displayed by Skeletal Muscles Lacking Bpag1 Crosslinking Proteins**

S. Lambert-Giroux,[1] C. Boudreau-Larivière[2]; [1]Human Development Program, Laurentian University, Sudbury, ON, Canada, [2]School of Human Kinetics, Biomolecular Sciences, Laurentian University, Sudbury, ON, Canada

Bullous pemphigoid antigen 1 (Bpag1) crosslinking proteins are thought to interact with microfilaments (MFs), intermediate filaments (IFs) and microtubules (MTs) thereby integrating the functions of these cytoskeletal networks in a variety of tissue types including contractile cells. The objective of the present study was to determine the impact of Bpag1-deficiency on the protein expression levels of MF, IF and MT subunits, and on the subcellular localization of these cytoskeletal elements in skeletal muscle. Western blot analyses revealed significant reductions in the levels of α-actin (40%) and α-tubulin (35%) detected in skeletal muscle homogenates from homozygous Bpag1-deficient mice compared to those obtained from wild-type littermates belonging to the *dystonia musculorum ( $dt^{Tg4}$)* transgenic line. Desmin protein levels remained largely unaffected. Alterations in α-actin and α-tubulin protein levels are likely the result of post-transcriptional events because the levels of their respective mRNAs, as assessed by RT-PCR, were found to be similar in wild-type and Bpag1-deficient skeletal muscle. The subcellular localization of MFs, desmin IFs and MTs was assessed by fluorescence microscopy using longitudinal sections of hindlimb skeletal muscles and antibodies targeting desmin and α-tubulin and rhodamine-conjugated phalloidin to visualize the MF network. A significant number of fibers from the Bpag1-deficient muscles (70%; 150 fibers analyzed) displayed loss of desmin Z-line staining. Attenuated sarcoplasmic, subsarcolemmal and perinuclear α-tubulin staining was also observed in more than 50% (100 fibers analyzed) of muscle fibers lacking Bpag1. Finally, uneven actin sarcoplasmic staining patterns with a dramatic loss of staining within the subsarcolemmal compartment were documented for approximately 70% (250 fibers analyzed) of Bpag1-deficient fibers. Together, the findings suggest that these cytoarchitectural modifications are likely implicated in the development of the post-natal skeletal myopathy that occurs in homozygous *dt* mice. Supported by NSERC (Canada)

2360

### Regulation of RLC Phosphorylation by $Ca^{2+}$ Transients During Myofibrillogenesis

J. D. Eskew, M. Terry, H. Kalluri, M. B. Ferrari; School of Biological Sciences, University of Missouri - Kansas City, Kansas City, MO

Intracellular $Ca^{2+}$ transients regulate myofibrillogenesis. While a link between these physiological signals and sarcomere assembly is well-established, the molecular and biochemical links have not been elucidated. Multiple downstream effectors have been proposed, but involvement of the $Ca^{2+}$/CaM/MLCK/RLC cascade in regulating myosin thick filament assembly in embryonic skeletal and cardiac myocytes has the most experimental support. We are attempting to distinguish between two different models: that only MLCK activity (i.e. RLC phosphorylation) is required for myosin thick filament assembly, versus more complicated assembly kinetics involving both MLCK and myosin phosphatase (PP1M) activities. We are using ryanodine to block transients, ML-7 and pseudosubstrate peptides to inhibit MLCK, and tautomycin or M-subunit specific morpholinos to knock down PP1M activity. In addition, we are using caffeine superfusion to test the role of specific $Ca^{2+}$ transient parameters in regulating RLC phosphorylation. Extracts from treated myocytes are compared with paired control extracts using fluorescence 2D differential gel electrophoresis (DIGE). Spots of interest are cut from replicate silver-stained gels for MS identification and phosphopeptide analysis. Several RLC spots show significant pI shifts consistent with differences in phosphorylation state; these shifts are consistent with expectations. For example, ryanodine reduces while tautomycin increases the density of several acidic RLC spot variants, consistent with increased and decreased phosphorylation, respectively. When combined with cell physiological approaches (e.g. superfusion), these powerful proteomics methods enable the discovery and description of biochemical changes resulting from $Ca^{2+}$ transient production. Supported by NIH RO1 AR47579 to M.B.F. .

2361

### FRET Imaging of Z-band Proteins in Living Skeletal Muscle Cells

A. L. Stout, J. Wang, J. M. Sanger, J. W. Sanger; Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA

In recent years, a large number of proteins associated with Z-bands in skeletal and cardiac muscle have been identified, but the three-dimensional arrangements of most of these proteins in immature and mature myofibrils are largely unknown. By combining sensitized emission FRET (Fluorescence Resonance Energy Transfer) imaging with quantitative analysis techniques, we are gaining new information about these arrangements that complements existing data obtained from in vitro assays such as yeast 2-hybrid experiments. Skeletal myotubes cultured from quail embryos were transfected to co-express CFP and YFP fusions with alpha-actinin, FATZ, telethonin, and myotilin in various pairwise combinations The FRET response, expressed as a percent efficiency, E%, was measured in Z-bodies of premyofibrils and nascent myofibrils and Z-bands of mature myofibrils. Multiple linear regression analysis was used to identify changes in E% that were correlated with CFP location on the protein (C- or N-terminus) after controlling for possible effects of other factors such as fusion protein expression level. Where relevant, we also used this approach to search for changes in E% correlated with the maturity of the myofibril (Z-body or Z-band). For example, in cells coexpressing CFP-FATZ and YFP-alpha-actinin, we found that in Z-bodies, E% was unaffected by the location of CFP while in Z-bands, E% was systematically higher when CFP was on the N-terminus of FATZ than when it was on the C-terminus. This result suggests that the conformation and/or flexibility of the FATZ molecule may change during myofibrillogenesis, possibly because of new protein-protein interactions that arise as Z-bands mature. Similar work was done with the myotilin/alpha-actinin pair, the FATZ/telethonin pair, and the FATZ/myotilin pair. Using our results and published results of in vitro assays, we have constructed a simple model describing the arrangement of these four proteins in Z-bands.

2362

### Myofibrillogenesis in Skeletal Muscle Cells in Zebrafish

J. W. Sanger, A. Du, B. Holloway, J. Wang, J. M. Sanger; Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA

The premyofibril model that we have proposed to explain myofibrillogenesis suggests that the formation of mature myofibrils is preceded by two intermediary structures: premyofibrils and nascent myofibrils (Du et al., 2003; Sanger et al., 2005). To determine if this model applies to zebrafish, we stained developing embryos at 20 - 25 hours post-fertilization with antibodies to sarcomeric alpha-actinin and muscle myosin II. In the developing somitic muscle the proteins were localized in fibrils in patterns that appeared identical to those in fibrils detected in avian muscle cultures, i.e. premyofibrils, nascent myofibrils and mature myofibrils., Premyofibrils staining positively with phalloidin and with anti-alpha-actinin antibodies extended from end to end in elongated cells in the youngest somites. In older somites with mature myofibrils, premyofibrils were present at the ends of the cells, suggesting that as the cells grew longer, premyofibrils were involved in the elongation of myofibrils that accompanied cell growth. We assembled a plasmid encoding YFP-sarcomeric alpha-actinin with a zebrafish skeletal muscle alpha-actin promoter. Embryos

microinjected with this plasmid expressed YFP-alpha-actinin near the end of one day in Z-bodies and Z-bands in the skeletal muscle cells along the flank of the zebrafish. On day six post-fertilization myofibrils with YFP-alpha-actinin incorporated in Z-bands were visible in the mosaic transgenic zebrafish. The expression of this probe did not affect the contraction of mature myofibrils in the zebrafish. The dynamics of YFP-alpha-actinin measured in the Z-bands with FRAP (Fluorescence recovery After Photobleaching) analysis showed a recovery profile similar to those found for YFP-alpha-actinin dynamics in avian skeletal muscle Z-bands (Wang et al., 2005). These observations indicate that the zebrafish system will allow the details of myofibril formation to be examined directly in a live animal.

2363

**Targeted Disruption of N-RAP Gene Function by RNA Interference: A Role for N-RAP in Myofibril Organization**

A. Dhume, R. Horowits; National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD

N-RAP is a muscle-specific protein concentrated in myofibril precursors during sarcomere assembly. We used RNA interference to achieve a targeted decrease in N-RAP transcript and protein levels in primary cultures of embryonic mouse cardiomyocytes. N-RAP protein knockdown was associated with decreased myofibril assembly, as assessed by alpha-actinin organization into mature striations, while cell spreading was not affected. The effect of N-RAP knockdown on alpha-actinin organization was partially rescued by expression of N-RAP-LIM-IB, a deletion mutant containing the N-RAP LIM domain and simple repeats, but omitting the super repeats. This construct allows normal assembly of alpha-actinin into mature striations, but disrupts sarcomeric actin organization (Carroll et al. *J Cell Sci 117:* 105-114, 2004). N-RAP-LIM-IB expression prevented N-RAP knockdown by siRNA at both the mRNA and protein levels. Therefore, the mechanism by which N-RAP-LIM-IB rescues myofibril assembly is likely to be by its affect on endogenous N-RAP expression. Transcripts encoding the N-RAP binding proteins alpha-actinin, Krp1, and MLP were expressed at normal levels during N-RAP knockdown, and alpha-actinin and Krp1 protein levels were also unchanged. Transcripts encoding muscle myosin heavy chain and nonmuscle myosin heavy chain IIB were also expressed at relatively normal levels. However, decreased N-RAP protein levels were associated with dramatic changes in the encoded myosin proteins, with muscle myosin heavy chain levels increasing and nonmuscle myosin heavy chain IIB decreasing. N-RAP transcript and protein levels recovered to normal by days six and seven, respectively, and the changes in myofibril organization and myosin heavy chain isoform levels were reversed. Our data indicate that myofibril assembly is closely linked to N-RAP protein levels, and that N-RAP protein levels regulate the balance between nonmuscle myosin IIB and muscle myosin by post-trancriptional mechanisms.

2364

**Expression of Muscle-specific Calpain, p94/calpain3, at The N2A and M-line Regions of Connectin/titin during Myogenesis**

K. Ojima,[1,2] H. Sorimachi[1,2]; [1]Department of Enzymatic Regulation for Cell Functions, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, [2]CREST, JST, Kawaguchi, Japan

Calpains comprise a large family of intracellular $Ca^{2+}$-requiring cysteine proteases, which cleave their substrates at limited and specific sites to modulate their functions, thus called “modulator proteases”. p94/calpain3 is predominantly expressed in skeletal muscles and the defect of its proteolytic activity causes limb-girdle muscular dystrophy type 2A. Furthermore, p94 binds to connectin/titin at the N2A and M-line regions shown by yeast two-hybrid studies, suggesting that p94 plays significant roles as a myofibrillar structural protein as well as a protease. However, it is unclear when p94 is localized to the specific regions of connectin/titin during the assembly of sarcomere structure. In this study, we examined spatio-temporal expression of p94 during myogenesis in relation to that of connectin/titin. RT-PCR and western blot analyses revealed that p94 and its splicing variants were detected immediately after muscle differentiation, and that their amount increased during development. At the early stages of muscle differentiation, p94 was distributed throughout myocytoplasm without any specific localization. One week after differentiation, p94 was expressed in the N2A and M-line regions of connectin/titin. Our results indicate that p94 is incorporated into its proper positions on connectin/titin after sarcomere structures are fully matured.

2365

**Non-Sarcomeric Tropomyosin Isoforms Define Novel Filament Compartments in Skeletal Muscle Fibers**

N. E. Vlahovich,[1] S. Artap,[1] A. Kee,[1] G. Schevzov,[2] P. Gunning,[2] E. Hardeman[1]; [1]Muscle Development Unit, Children's Medical Research Institute, Westmead, Australia, [2]Oncology Research Unit, The Children's Hospital at Westmead, Westmead, Australia

Tropomyosins (Tms), a family of filamentous proteins consisting of over forty isoforms, play an important role in defining the function of actin filaments in both muscle and non-muscle cells. Isoforms are derived from four genes (alpha, beta, gamma and delta) and are produced through the use of alternative promoters and alternate RNA splicing. In non-muscle cells, Tm isoforms have been shown to be functionally distinct, playing roles in diverse processes such as cell growth, differentiation, cell division and vesicle transport. Using western blotting and immunofluorescence techniques with isoform-specific antibodies, we have identified a number of these non-muscle Tms in skeletal muscle fibers. These Tms were found to sort to distinct compartments in both developing and mature muscle cells. Tm5NM1, a product of the gamma gene, was found to localize to the sarcolemma and the Z-line associated cytoskeleton (Z-LAC). An antibody recognizing Tms 1, 2, 5b and 6, from the alpha and beta genes, demonstrated that these isoforms are present at the myotendinous junction. Tm4, from the delta gene, localizes to the myotendinous junction as well as the postsynaptic region of the neuromuscular junction. In addition, and surprisingly, Tm4 is present in a novel longitudinal pattern of immunofluorescence found in a subset of muscles including the extraocular, diaphragm and flexor digitorum brevis muscles. This unique pattern of Tm4 localisation can be induced in the soleus muscle in response to stretch suggesting that this structure is associated with tension or loading. These findings regarding Tm4 distribution may also provide a new classification of muscles based on extra-sarcomeric filament structures. Taken together, our observations suggest that different non-muscle Tms define distinct microfilament populations in muscle fibers.

2366

**Functional Interaction of Protein 41R and M-line Titin in Sarcomere Assembly**

A. Zhou, S. Huang, E. J. Benz; Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

A diverse array of red blood cell protein 4.1 (4.1R) isoforms is expressed in many non-erythroid tissues. In our previous work, we have shown that 4.1R localizes to the A-band of sarcomeres and associates with myosin, tropomyosin and actin. To further understand the role of 4.1R in skeletal

muscle sarcomere structure and function, yeast two hybrid assay and biochemical binding assay were used to characterize the binding ligands of 4.1R in skeletal muscle. We have further identified that 4.1R interacts with M-line titin. In this study, we attempted to understand the functional importance of the interaction between 4.1R and M-line titin. Immunofluorescent staining showed that 4.1R partially co-localized with M-line titin. Furthermore, an in vitro binding assay revealed that the amino acids encoded by exons 17-18 of 4.1R ($4.1R^{ex17-18}$) and the MIg9/10 domain of titin accounted for their interaction. To study the functional association of 4.1R and titin, we used adenovirally mediated gene transfers to over-express $4.1R^{ex17-18}$ in differentiating C2C12. In control GFP-infected myotubes, α-actinin and M8/9 titin assumed a striated distribution at the level of the Z-disk and M-line, respectively. However, over-expression of $4.1R^{ex17-18}$ altered the distribution and organization of M-line titin. The degree of M-line titin disruption correlated with expression levels of $4.1R^{ex17-18}$. Infected cells expressing low levels of $4.1R^{ex17-18}$ showed diffused M-line titin in the myoplasm with occasional striated periodicity in register with the M-line. Infected cells with high levels of $4.1R^{ex17-18}$ significantly reduced M-line titin striation and resulted in disorganized structures or large aggregates of titin in the myoplasm. These findings support the idea that the interaction of 4.1R with M-line titin may play a critical role in the assembly of M-line in striated muscle.

2367

**Opposing Effects of Two Single Amino Acid Point Mutations Within the Myosin Motor Domain**

C. M. Dambacher,[1] A. F. Knowles,[2] W. A. Kronert,[1] J. A. Suggs,[1] S. I. Bernstein[1]; [1]Biology Department and Molecular Biology Institute, San Diego State University, San Diego, CA, [2]Chemistry, San Diego State University, San Diego, CA

Mutations within contractile proteins can engender various phenotypic abnormalities including skeletal muscle hypercontraction and cardiac hypertrophy. We investigated the *in vivo* and *in vitro* properties of two homozygous-viable *Drosophila* myosin mutants, *D45* (A261T) and $Mhc^5$ (G200D). In the case of the *D45* mutation, the single amino acid change resides near β7 (this β-strand and switch-2 flank a region which has been shown to move as a solid body), while the $Mhc^5$ mutation is located at the base of loop 1. Both regions have influence in regulating the rate of nucleotide exchange from the ATP-binding pocket. The mutation *D45* was originally identified as a suppressor of the troponin I (TnI) $hdp^2$ mutation, which causes muscle hypercontraction, resulting in a wings-up phenotype. The $Mhc^5$ mutation is lethal when co-expressed with $hdp^2$, presumably enhancing the hypercontraction phenotype. Ultrastructural analysis of flies expressing MHC containing the suppressor (*D45*) mutation revealed wild-type assembly and stability of myofibrils, while flies expressing myosin with the enhancer mutation ($Mhc^5$) display a severe hypercontraction phenotype visible by two days post-eclosion. Myosin containing the *D45* suppressor mutation has decreased $Ca^{2+}$ ATPase and reduced basal and actin-stimulated $Mg^{2+}$ ATPase as compared to the wild-type indirect flight muscle isoform (IFI). Myosin containing the $Mhc^5$ enhancer mutation showed a greater than two-fold increase in basal $Mg^{2+}$ but similar actin-stimulated $Mg^{2+}$ ATPase as the IFI. The average actin sliding velocity was reduced by two-fold for *D45* myosin, while $Mhc^5$ myosin shows a 15% increase in velocity of actin filament translocation as compared to the IFI. These results suggest that suppression of the TnI hypercontraction phenotype is accomplished by expression of a less-active MHC, while onset and enhancement of muscle hypercontraction can be achieved with expression of an overactive MHC isoform.

2368

**Troponin I Heptad Hydrophobic Repeats (HR): Effect on Calcium Sensitivity of Vertebrate Striated Muscle Contraction**

M. Mukherjea, T. Chaudhuri, J. Randall, S. Sarkar; Anatomy & Cell Biology, Tufts University, Boston, MA

In vertebrates, striated muscle contraction is a $Ca^{2+}$ regulated process which is mediated through troponin (Tn). The Tn complex consists of three different proteins: troponin C (TnC) which binds $Ca^{2+}$, troponin I (TnI) which binds to actin and inhibits acto-myosin interaction, and troponin T (TnT) which attaches Tn to Tm. Among the inter-subunit interactions in the Tn complex, the binary interaction between TnI and TnT as well as its effect on Tn structure and function are not clearly understood. We previously identified and proposed that an evolutionarily conserved HR domain present in both TnI and TnT may be involved in a coiled-coil TnI-TnT heterodimer formation. To study the function of the TnI HR domain, we designed both point and deletion mutations in the HR and tested their influence on TnI-TnT binary interactions. Using different in vitro approaches like yeast-two-hybrid and affinity measurements, we observed that the TnI HR deletion mutants showed 60-70% lesser activity than the wild-type whereas the point mutations showed comparable activity, indicating that the HR domains are involved in TnI-TnT binary interaction. To characterize the biological function of HR motifs in TnI and the functional parameters of TnI-TnT interaction we tested how they regulate the $Ca^{2+}$ sensitivity of muscle contraction. We performed a reconstituted actomyosin ATPase assay to calculate the ATP hydrolysis rate of the myosin motor. We also over-expressed the wild-type and mutant TnIs in mouse myoblast cells and studied the effect of the different HR mutations on the efficiency of the myosin ATPase. Our results show that disruption of the TnI-TnT structural interaction, especially by the deletion mutants, inhibit the formation of the ternary Tn complex and significantly decreases the $Ca^{2+}$ sensitivity of the thin filament during striated muscle contraction.

2369

**Troponin T: Role of the Fetal and α/β Exons on Calcium Sensitivity in Vertebrate Fast Skeletal Muscle during Development**

T. Chaudhuri, M. Mukherjea, S. Sachdev, S. Sarkar; Anatomy & Cell Biology, Tufts University, Boston, MA

In mammalian fast skeletal muscle, constitutive and alternative splicings from a single troponin T (TnT) gene produce multiple developmentally regulated and tissue specific TnT isoforms. Two exons, α and β, coding for two different 14 amino acid peptides are spliced in a mutually exclusive manner giving rise to the adult TnTα and the fetal TnTβ isoforms. In addition, an acidic peptide coded by a fetal (f) exon is specifically present in TnTβ and absent in the adult isoforms. To define the functional role of the f and α/β exons, we constructed different TnT cDNAs from a single human fetal fast skeletal TnTβ cDNA clone to circumvent the problem of N-terminal sequence heterogeneity present in wild-type TnT isoforms, irrespective of the stage of development. Nucleotide sequence of these constructs, viz. TnTα, TnTα+f, TnTβ-f and TnTβ are identical, except for the presence or absence of the α or β and f exons. Our results, using the recombinant TnT isoforms in different functional *in vitro* assays, show that the presence of the f peptide has a strong inhibitory effect on binary interactions between TnT and other thin filament proteins, TnI, TnC and tropomyosin. Tn complexes reconstituted with the four TnT isoforms showed different patterns of $Ca^{2+}$ sensitivities in the actomyosin-ATPase assay. The presence of the f peptide also led to reduced $Ca^{2+}$ dependent ATPase activity in the myofilament assay whereas the contribution of the α and β peptides in the biological activity of TnT was primarily modulatory. These results indicate that the f peptide confers an inhibitory effect on the biological function of fast skeletal TnT and this can be correlated with changes in the $Ca^{2+}$ regulation associated with development in fast skeletal muscle.

2370

**Obscurin Depletion Inhibits Sarcomeric Myosin Integration and Thick Filament Organization in Skeletal Myofibrils of Developing Zebrafish Embryos**

M. O. Raeker,[1] A. Kontrogianni-Konstantopoulos,[2] S. B. Sutter,[1] A. B. Borisov,[1] R. J. Bloch,[2] S. E. Lyons,[3] M. W. Russell[1]; [1]Pediatrics, University of Michigan, Ann Arbor, MI, [2]Physiology, University of Maryland, Baltimore, MD, [3]Internal Medicine, University of Michigan, Ann Arbor, MI

Obscurin is a giant (~800 kD) sarcomere-associated protein whose interactions with titin and ankyrin suggest that it may have an important role in the assembly and structural support of striated myofibrils. To examine the potential role of obscurin during skeletal myofibrillogenesis *in vivo*, morpholino antisense oligonucleotides targeting the obscurin translation initiation site (MO1), the 5'UTR (MO2) or a corresponding control morpholino (5 base pair mismatch to MO1 or MO2) were injected into zebrafish embryos (1-4 cell stage). Injection of either MO1 or MO2 resulted in diminished obscurin expression and significant developmental defects including decreased numbers and marked disarray of skeletal myofibrils compared to controls. At the level of the sarcomere, obscurin depletion appeared to selectively disrupt M band assembly and thick filament organization; Z band architecture and spacing were preserved as documented by a normal pattern of α-actinin immunolocalization. Sarcomeric myosin localized diffusely in the skeletal muscle of the MO1- and MO2-injected embryos and was not organized into well-formed A bands. Interestingly, depletion of obscurin also caused an alteration in its own cellular distribution. Unlike control embryos in which obscurin localized primarily to the M band with some Z band staining, in MO1- and MO2-injected embryos, the M band localization was markedly diminished while the Z band staining was preserved. These results support a critical role for obscurin in the assembly and organization of the myosin thick filament array during vertebrate development. Furthermore, the diminished capacity to generate mature striated myofibrils in response to obscurin depletion suggests that it may have a vital role in the causation of or adaptation to cardiac and skeletal myopathies.

2371

**Localization of Obscurin in Skeletal Muscle Fibers**

A. L. Bowman, A. Kontrogianni-Konstantopoulos, D. H. Catino, H. Gonzalez-Serratos, R. J. Bloch; Physiology, University of Maryland, Baltimore, Baltimore, MD

Obscurin is a giant sarcomeric protein that is critical in the development of the A band and M line in skeletal muscle. Unlike nebulin and titin, obscurin surrounds myofibrils at the level of the M and Z lines. Our laboratory has generated antibodies to different regions of obscurin, the amino terminus, the carboxy terminus, and the Rho-GEF domain. All recognize the ~800 kDa form of obscurin in western blots of skeletal muscle, but they differ in their ability to bind smaller isoforms of obscurin. All three antibodies also recognize smaller bands, between ~75 and 200 kDa, but yield different patterns of these smaller bands. To assess the relationship of obscurin to the Z-disk, we used these antibodies to label extensor digitorum longus muscles stretched to sarcomere lengths ≥3.0μm. Immunofluorescence images revealed a doublet flanking the Z-disk of sarcomeres in stretched samples, but only single lines at Z-disks of sarcomeres at normal resting lengths (~2 μm). Preliminary analyses of the distances between the labeled structures in obscurin and the middle of the nearest Z-line suggest that the antibodies to the Rho-GEF domain label structures that are farther from the Z-line than antibodies to the carboxy terminus. Since these domains should be close enough to each other in the ~800 kDa form of obscurin to be indistinguishable by confocal microscopy, we hypothesize that the antibodies to the Rho-GEF domain selectively label one or more of the smaller isoforms of obscurin, that concentrate at distinct sites with respect to sarcomeres that separate from the Z-disk more readily upon stretching. Supported by a training stipend (T32 GM08181, to ALB), grants from the NIH (RO1 HL64304 and the Muscular Dystrophy Association (to RJB) and a Development Award to AKK from the Muscular Dystrophy Association.

2372

**Expression of Tropomodulin1 in the Heart Rescues Embryonic Lethality of Tropomodulin1 Null Mice**

R. B. Nowak,[1] J. Moyer,[1] K. Fritz-Six,[1] C. Ferreira-Mota,[1] M. A. Sussman,[2] V. M. Fowler[1]; [1]Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Biology, San Diego State University, San Diego, CA

Tropomodulin1 (Tmod1) caps the pointed ends of actin filaments in striated muscle sarcomeres and in the erythrocyte membrane skeleton, and is first expressed at E7.5 of embryogenesis in myocytes of the developing heart tube and in blood islands of the yolk sac. Targeted deletion of the Tmod1 gene in mice leads to abnormal cardiac development, and defective vasculogenesis and hematopoesis in the yolk sac, followed by embryonic lethality between E9.5-10. We rescued the embryonic lethality of the Tmod1 knockout mouse by crossing with a TOT mouse that expresses a Tmod1 transgene in the heart under the control of the α-myosin heavy chain promoter. Genotyping of litters from crosses of Tmod1-/+;TOT+ mice with Tmod1+/- mice demonstrates that embryos with no endogenous Tmod1 but expressing the Tmod1 transgene (Tmod1-/-;TOT+) develop to term and that pups are viable and fertile. Western blotting and immunofluorescence staining demonstrates that Tmod1 protein is present only in the heart but not the blood cells of Tmod1-/-;TOT+ rescued embryos. In contrast to Tmod1 null embryos, which fail to undergo normal looping morphogenesis of the heart tube and have defective yolk sac vasculogenesis, the cardiac and yolk sac morphology of Tmod1-/-;TOT+ embryos are similar to wild type embryos. Immunofluorescence staining and confocal microscopy of hearts of E8-9.5 embryos demonstrates that myofibril assembly is grossly defective in the absence of Tmod1 but normal in Tmod1-/-;TOT+ embryos. We conclude that 1) Tmod1 expression in blood cells is not required for embryonic development or viability, 2) the absence of Tmod1 in blood cells does not lead to cardiac defects, 3) Tmod1 function in cardiac myocytes is directly required for cardiac development and function, 4) yolk sac defects are secondary to defects in cardiac development.

2373

**The Role of the Serine Rich Linker Regions of Nebulette and Nebulin in Myofibril Assembly**

Z. Panaviene,[1] K. J. Doane,[2] C. L. Moncman[1]; [1]Biochem, University of Kentucky, Lexington, KY, [2]Anatomy, Northeastern Ohio Universities College of Medicine, Rootstown, OH

Nebulette and nebulin are large modular proteins associated with the thin filament-Z-line complex of striated myofibrils. Both proteins contain an acidic N-terminal region, a large central repeat domain, a serine rich linker region and a C-terminal SH3 domain. We have previously demonstrated using nested deletions of GFP tagged nebulette constructs that the linker region was necessary for proper targeting of the repeat domain to the Z-lines. These data suggest that the linker region plays an important role in the assembly and stability of nebulette with the Z-lines. The recent

identification of the focal adhesion splice variant LIM-nebulette, that lacks the majority of the linker region supports this hypothesis. Expression of a nebulette cDNA containing the C-terminal splice variant of the LIM-nebulette or a nebulette cDNA completely lacking the linker region result in a membrane skeleton distribution of the recombinant protein. We have extended these studies by examining the ability of the nebulin and LASP1 linker regions to perform similar targeting functions in muscle and nonmuscle cells and have found that both linker regions perform targeting functions in their perspective cell types. These data further support an essential role for the linker regions of these proteins in their assembly and function. We are currently performing a yeast two hybrid screen to identify interacting proteins for the linker region.

2374

**The N-terminal Domain of the Cardiac Muscle Protein Nebulette Interacts with a Number of Different Proteins**

W. B. Holmes, C. L. Moncman; Biochemistry, University of Kentucky, Lexington, KY

Nebulette is a member of the nebulin family of proteins found only in cardiac muscle. Nebulette has the same domain layout as nebulin along with ~ 70% sequence homology. However, nebulette is much smaller than nebulin, ~ 110 kD for nebulette vs. 600 - 900 kD for nebulin. The N-terminal domains of nebulette and nebulin are highly acidic with pIs of 3.9 and 4.9 respectively. The N-terminal domains of both proteins are well conserved in animals. Tropomodulin interacts with repeat modules at the N-terminal end of nebulin however no function has been identified for the acidic N-terminal domain of nebulin or nebulette. The smaller size of nebulette indicates that interaction with tropomodulin is unlikely. To evaluate the functions of the nebulette N-terminal domain we have used the yeast two-hybrid system to screen a human heart cDNA library using a.a. residues 1-80 as bait. We have identified 15 unique interactions using the two-hybrid system, among these are: myosin binding protein-c, cyclin I, filamin, lin-7 and a number of proteins with unknown function that are present in the EST database from striated muscle. These results suggest a role for the N-terminal domain of nebulette in mediating nebulette interactions with many other proteins.

2375

**A Frequency of Calcium Oscillation Determines a Rate of Sarcomere Assembly in C2C12 Myotubes**

H. Fujita, T. Nedachi, M. Kanzaki; TUBERO, Tohoku University, Sendai, Japan

The assembly of sarcomere, the smallest contractile unit in striated muscle, is a complex and highly coordinated process that relies on spatial organization of numerous sarcomeric proteins. Recent finding has indicated that spontaneous $Ca^{2+}$ transient is important for sarcomere development, but the precise mechanism underlying this event is still unknown. In order to investigate relationship between $Ca^{2+}$ transients and sarcomere assembly, we took advantage of electrical pulse stimulation (EPS) that allows us to control the frequency of $Ca^{2+}$ transients through depolarizing the membrane potential. At 6 days after inducing differentiation of C2C12 myoblasts, they formed myotubes and expressed whole set of sarcomeric proteins required for $Ca^{2+}$-induced contraction, but they did not display any contractile activity in response to EPS because sarcomere architecture was not yet developed. However, EPS-induced repetitive $Ca^{2+}$ transients (1 Hz) for 2 hrs induced a marked increase in their contractile activity that occurred in concurrent with the development of sarcomere structure. Application of low frequency of EPS (0.1 Hz) required much longer period until the immobile myotubes acquire contractile activity. A persistent increase in intracellular $Ca^{2+}$ (not oscillatory) by high frequency of EPS (10 Hz) was incapable of inducing sarcomere assembly. We have also found that EPS-induced $Ca^{2+}$ transients increased calpain-mediated proteolysis of talin and its prevention by calpain inhibitor abrogated the EPS-facilitated assembly of functional sarcomere. In addition, modulation of integrin signals by adding collagen or RGD-peptide significantly affected the EPS-induced acquisition of contractile activity. Together, these results indicate that a period required for the development of functionally active sarcomere structure is dependent on a frequency of $Ca^{2+}$ oscillation. Our results also suggest an importance of the reorganization of the integrins/talin /α-actinin complex mediated through the calpain-induced talin cleavage.

## Kinesin II (2376-2392)

2376

**Viability in Cells with an Activated Dicentric Chromosome Requires the Central Spindle and Kar3p**

J. N. Molk, J. Haase, E. D. Salmon, K. Bloom; Biology, University of North Carolina, Chapel Hill, NC

Dicentric chromosomes undergo a breakage-fusion-bridge cycle *in vivo.* In budding yeast, activation of a conditional dicentric chromosome is lethal in the absence of the DNA repair gene *RAD52*. Defects in proteins that weaken kinetochore-microtubule attachments partially suppress the *rad52* lethality in strains that contain activated dicentric chromosomes. We investigated if the central spindle contributes to breakage and/or resolution of the dicentric chromosome. To weaken the central spindle, we used *ase1Δ* mutants. Ase1p localizes to the spindle midzone and is required for spindle integrity during anaphase. Unexpectedly, the viability of cells with an activated dicentric chromosome in *ase1Δ* was decreased and was similar to deletion of *RAD52*. Therefore, central spindle function is required for cells to survive following activation of the dicentric chromosome and is separable from kinetochore function. In support of this hypothesis, the loss of several microtubule plus-end binding proteins (Bik1p, Slk19p, Cik1p, and Kar3p) resulted in low viability in cells with an activated dicentric chromosome. Bik1p and Slk19p have been implicated in maintaining spindle integrity where microtubule plus-ends overlap. To determine if Kar3p could act in a similar manner, we imaged Kar3p-GFP and found that Kar3p localizes to the mitotic spindle in early to mid-anaphase. We propose Kar3p, associated with Cik1p, functions at the plus-ends of overlapping microtubules in the spindle midzone to provide structural support during anaphase. In the presence of an activated dicentric chromosome, inward forces pulling the spindle poles together promotes spindle disassembly. Kar3p and other proteins at the plus-end in the central spindle may resist this force to allow recovery from the dicentric arrest.

2377

**Kar3 Dimerization with Cik1 Enhances Motor Velocity**

H. M. Annie Chu,[1] M. Yun,[2] D. E. Anderson,[1] H. Sage,[3] H. W. Park,[2] S. A. Endow[1]; [1]Cell Biology, Duke University, Durham, NC, [2]Structural Biology, St. Jude Children's Research Hospital, Memphis, TN, [3]Biochemistry, Duke University, Durham, NC

Kar3, a Kinesin-14 microtubule motor of *S. cerevisiae* required for mitosis and karyogamy, or nuclear fusion after mating, reportedly interacts *in vivo* with Cik1, a nonmotor protein, through its central, predicted coiled-coil domain. Although other workers have reported that neither Kar3 nor

Cik1 forms homodimers *in vivo*, we show here that purified Kar3 is a dimer *in vitro*, as assayed by sedimentation equilibrium ultracentrifugation. The protein appears as paired particles by rotary shadow electron microscopy (EM) and circular dichroism (CD) spectroscopy of the Kar3 stalk shows helical characteristics, indicating that dimerization of the protein probably occurs by coiled coil formation. CD analysis of the nonmotor region of purified Kar3/Cik1 protein indicates even higher helicity than observed for the Kar3 stalk, and, strikingly, rotary shadow EM reveals a large single or two smaller particles attached to a stalk, which is not visible in Kar3. Analytical ultracentrifugation shows that Kar3/Cik1 is a dimer at low concentration (2.58 μM). The Kar3/Cik1 motor moves on microtubules at a velocity of 2-2.4 μm/min, 2-5 fold faster than Kar3, and destabilizes microtubules at the lagging ends. Thus, structural changes in Kar3 occur upon dimerization with the Cik1 nonmotor protein that convert it into a faster motor. These changes are likely to regulate Kar3 motor activity *in vivo.*

2378

**Kidins220/ARMS-Containing Vesicles are Transported to the Central Region of Growth Cones through Complexes with KIF3A, KIF3C and KAP3A**

Y. H. Liao, W. W. Liu, S. M. Hsu, P. H. Huang; Graduate Institute of Pathology, National Taiwan University College of Medicine, Taipei, Taiwan Republic of China

In polarized neurons, kinesin superfamily proteins (KIFs) are the motors responsible for targeting membranous or non-membranous cargos to axons and dendrites. Among them, KIF3C, when associated with KIF3A, is proposed as a membranous vesicle-associated motor. However, the cargos for the KIF3A/3C complex have not been identified. Kidins220 (kinase D-interacting substrate of 220kD), also known as ARMS (ankyrin repeat-rich membrane spanning), accumulates at the tips of growing axons in cultured neurons and those of NGF-treated PC12 cells, but the molecular mechanism for such specific localization remains unknown. Here, we demonstrate a direct interaction of Kidins220 with KIF3C, KIF3A, and KAP3A in neurons, but not with KIF3B. Using yeast two-hybrid screening and co-immunoprecipitation assay, we identify that the C-terminal region of Kidins220 is essential for the interactions. By using in vitro cultured neuronal cells and in vivo sciatic nerve ligation experiments, we show that Kidins220 is anterogradely transported along axons and is focally enriched in the central region of growth cones. Moreover, by RNAi approach against KIF3 complex and the expression of dominant-negative KIF3A/KIF3C, the targeting of Kidins220 to the tips of neurite is inhibited. Furthermore, the expression of Kidins220ΔC construct that disrupts Kidins220-KIF3 binding interferes with the localization of Kidins220 at the tips of neurite in PC12 cells. The above evidence indicates that the targeting of Kidins220 to the tips of neurites depends exclusively on its C-terminal region and partially on the presence of KIF3A, KIF3C and KAP3A. By electron microscopy, Kidins220 is associated with 100 to 220 nm, electron-lucent, membranous vesicles. Taken together, our studies indicate that Kidins220 resides in membranous vesicles and targets to the tips of neurites through interaction with KIF3A/KIF3C/KAP3A complex.

2379

**The KIF3 Motor Transports N-cadherin and Organizes the Developing Neuroepithelium**

J. Teng,[1] T. Rai,[1] Y. Tanaka,[1] Y. Takei,[1] T. Nakata,[1] M. Hirasawa,[2] A. B. Kulkarni,[2] N. Hirokawa[1]; [1]Department of Cell Biology and Anatomy, University of Tokyo Graduate School of Medicine, Tokyo, Japan, [2]Functional Genomics Section, Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD

In the developing brain, the organization of the neuroepithelium is maintained by a critical balance between proliferation and cell−cell adhesion of neural progenitor cells. The molecular mechanisms that underlie this are still largely unknown. Here, through analysis of a conditional knockout mouse for the *Kap3* gene, we show that post-Golgi transport of N-cadherin by the KIF3 molecular motor complex is crucial for maintaining this balance. N-cadherin and beta-catenin associate with the KIF3 complex by co-immunoprecipitation, and colocalize with KIF3 in cells. Furthermore, in KAP3-deficient cells, the subcellular localization of N-cadherin was disrupted. Taken together, these results suggest a potential tumour-suppressing activity for this molecular motor.

2380

**Functional Coordination of Intraflagellar Transport Motors in *C elegans* Neurons**

G. Ou,[1] O. E. Blacque,[2] J. J. Snow,[1] M. R. Leroux,[2] J. M. Scholey[1]; [1]Center for Genetics and Development, Section of Molecular & Cellular Biology, University of California, Davis, Davis, CA, [2]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

Cilia play diverse roles in motility and sensory reception, and defects in their functions contribute to ciliary diseases. Intraflagellar transport (IFT) motors assemble and maintain cilia by transporting ciliary precursors, bound to protein complexes called IFT particles, from the base of the cilium to their site of incorporation into ciliary structures. In *Caenorhabditis elegans,* this is accomplished by two IFT-motors, kinesin-II and OSM-3 kinesin, which are both members of the kinesin-2 family. These motors cooperate to form two sequential anterograde IFT pathways, the “middle” and “distal” segment pathways, that build distinct parts of cilia. By watching fluorescent IFT-motors and IFT-particles move along the cilia of numerous ciliary mutants, we identified three ciliary proteins that mediate the functional coordination of these motors. The Bardet-Biedl Syndrome (BBS) proteins, BBS-7 and BBS-8 are required to stabilize complexes of IFT-particles containing both IFT-motors because in *bbs-7 and bbs-8* mutants, IFT-particles break down into two subcomplexes, A and B, which are moved separately by kinesin-II and OSM-3-kinesin, respectively. A novel, conserved ciliary protein, DYF-1, is specifically required for OSM-3-kinesin to dock onto and move IFT-particles, since in *dyf-1* mutants, OSM-3-kinesin is inactive and intact IFT-particles are moved by kinesin-II alone. These findings implicate BBS ciliary disease proteins and a novel kinesin-2 activator in the formation of two IFT pathways that build functional cilia. Similar screens of ciliary mutants are being used to identify cargo molecules as well as additional regulatory proteins that mediate the functional coordination of these IFT-motors.

2381

**The Roles of Kinesin Motor Proteins in Spermatid Differentiation in *Marsilea vestita***

F. Deeb, S. M. Wolniak; Cell Biology and Molecular Genetics, University of Maryland, College Park, MD

The microspore of the water fern *Marsilea vestita* contains a single cell that undergoes a series of nine successive mitotic divisions to produce 32 spermatids, six sterile jacket cells and one prothallial cell. After the dry microspore is placed into water, cytoplasm reorganization precedes the first

division; certain proteins and mRNAs aggregate into zones that later become the spermatogenous initials of the gametophyte. Our results indicate that some proteins and some mRNAs become redistributed by kinesin-driven movements along cytoplasmic microtubules. Gametophyte development relies on the translation of stored mRNAs, and we developed RNAi strategies to target the degradation of specific mRNAs to arrest development. These published studies show that the temporal and spatial patterns of translation in the gametophyte are precisely ordered. Centrin translation occurs exclusively in spermatogenous cells and is required for the *de novo* formation of basal bodies. Various tubulin isoforms become localized in the spermatogenous cells and are essential for nuclear shaping and ciliogegnesis. We are studying how different kinesins affect development, cell fate, and differentiation during spermiogenesis. dsRNA probes were made from cDNAs encoding seven different kinesin isoforms isolated from our gametophyte library. The dsRNAs were added to populations of developing gametophytes to assess the time and stage of development at which each transcript becomes limiting. Two kinesin knockdowns show no effects on development while the others alter division patterns at specific developmental stages. The disruption of the pattern of cell divisions in the gametophyte predicts changes in cell fate that are manifested by changes in centrin translation and β-tubulin localization patterns. The loss of kinesin-based movements at particular times during gametophyte development effectively results in changes in cell fate determination. (Supported by NSF grant MCB-0234423 to SMW).

2382

### A Truncated *Leishmania* K39 Kinesin Protein Binds Along Cortical Microtubules in an ATP-Dependant Manner and Accumulates at the Tip of the Cell

N. J. Gerald, D. M. Dwyer; Lab of Parasitic Diseases, NIAID, NIH, Bethesda, MD

Kinesins are a superfamily of microtubule motor proteins with diverse functions that include trafficking, signal transport and microtubule reorganization in eukaryotic cells. Patients with the parasitic disease visceral leishmaniasis mount a strong immune response against the *Leishmania* kinesin K39, indicating that parasites produce this protein during the course of infection. However, the role of K39 in the cell biology of *Leishmania* remains unknown. To investigate the function of this kinesin in L. donovani, we expressed GFP fusions to different K39 domains in promastigote cells. The motor domain (HEAD) localized diffusely throughout the cytoplasm, while the coiled-coil C-terminus (TAIL) localized to the anterior and posterior ends the cell. The motor domain combined with 65% of the coiled-coil tail (HCC) accumulated in a bolus at the posterior end of the cell near the tips of cortical microtubules. To test the association of these proteins with microtubules, cells were extracted with detergent to isolate the insoluble microtubule cytoskeletons. Western blots demonstrated that native K39, HEAD, TAIL, and HCC proteins were all largely solubilized with detergent. When ATP was depleted prior to detergent extraction, native K39 and HCC protein showed increased binding to microtubule cytoskeletons. The HEAD and TAIL proteins remained soluble after ATP-depletion. Microscopy of ATP-depleted cytoskeletons revealed that HCC was loaded prominently along the lengths of cortical microtubules leading to the posterior end of the cell. Microtubule associated HCC was released back into the supernatant with the addition of fresh ATP. These results are consistent with an ATP dependent kinesin motor that traffics along cortical microtubules to their tips at the posterior end of the cell.

2383

### K-165: An Organelle Motor in the Squid Giant Axon

J. A. DeGiorgis, P. E. Gallant, T. S. Reese, J. A. Galbraith; Laboratory of Neurobiology, National Institutes of Health, Bethesda, MD

Conventional kinesin, the founding member of a diverse and functionally important group of molecular motor proteins, was discovered in the North Atlantic Squid, *Loligo pealei*. While its role was attributed to moving vesicles along microtubules, little evidence links the motor activity of conventional kinesin to vesicle translocation. Here, we identify a second kinesin in squid by analysis of a nucleotide sequence from a new Expressed Sequence Tag (EST) database representing RNAs expressed in the squid stellate ganglia. A peptide polyclonal antibody raised against this new kinesin identified seven clones in an optic lobe expression library, each of which overlapped with the kinesin EST sequence. This antibody recognized an ~165-kDa band in immunoblots while anti-conventional kinesin antibodies recognize a band at 110 kDa. Mass spectroscopic analyses of the ~165 kDa (K-165) and 110 kDa (K-110) bands yielded peptides that matched the predicted amino acid sequence of the new kinesin and conventional kinesin respectively. Thus, this new kinesin (K-165) and conventional kinesin (K-110) are products of different genes and antibodies raised against these two kinesins are specific to their respective gene products. Further immunoblot analyses demonstrated that the K-165 protein is highly enriched in sucrose density gradient fractions of KI-washed axoplasmic organelles. The K-165 antibody also decorates axoplasmic organelles in extruded axoplasm by immuno-gold electron microscopy. These data demonstrate that there are at least two kinesins in the giant axon, that K-165 is tightly associated with organelles, and is a candidate to be an organelle motor.

2384

### $PIP_3$ Transport by GAKIN, a Kinesin 3 Family Protein, Regulates Neuronal Polarity

K. Horiguchi, T. Hanada, A. H. Chishti; Department of Pharmacology and UIC Cancer Center, University of Illinois, College of Medicine, Chicago, IL

Phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of PI 3-kinase, is an important second messenger implicated in signal transduction and membrane transport pathways. In hippocampal neurons, the accumulation of PIP3 at the tip of axon regulates the axon-dendrite specification and neuronal polarity formation. Here, we show that a kinesin-like protein GAKIN/KIF13B directly interacts with a PIP3-interacting protein, PIP3BP/Centaurin-alpha, and mediates the transport of PIP3-containing vesicles. The FHA domain of GAKIN mediates the interaction with PIP3BP. The interaction is specific for GAKIN; since human KIF13A, the closest homologue of GAKIN, does not bind to PIP3BP. Recombinant GAKIN and PIP3BP form a complex on synthetic liposomes containing PIP3, and supports the motility of the liposomes along microtubules *in vitro*. In differentiating PC12 cells, both GAKIN and PIP3BP localized at the tip of the growing neurites. PIP3, as monitored by GFP-PH domain of AKT (GFP-AKT-PH), was also found at the tip of the neurites. Overexpression of DN-GAKIN, which is the motor domain deleted construct, significantly reduced the accumulation of PIP3 at the neurite tips. In cultured hippocampal neurons obtained from mouse embryo, PIP3 accumulation was observed at the tip of axons. Overexpression of DN-GAKIN inhibited the accumulation of PIP3. Importantly, heterologous expression of DN-GAKIN in hippocampal neurons induced morphological changes with highly branched dendrites, and resulted in the loss of axonally differentiated neurites as determined by tau-1 monoclonal antibody staining. Together, these results suggest that GAKIN- PIP3BP complex mediates the transport of PIP3 containing vesicles in neuronal cells and contributes to the accumulation of PIP3 at the axon tip, which in

turn regulates the axon-dendrite specification and neuronal polarity formation *in vivo*.

2385

**Localization and Expression of Kinesin Motor Proteins in Cancer Cells**

K. M. Bartoli, W. S. Saunders; Biochemistry and Molecular Genetics, University of Pittsburgh, School of Medicine, Pittsburgh, PA

Kinesin motor proteins play an important role in transportation of specific intracellular cargoes along microtubules. Some kinesins have been implicated in mitosis and are important for proper cell division. Recent studies suggest that some motor proteins are mislocalized as well as overexpressed in cancer cells leading to mitotic defects. This led us to systematically compare the expression and distribution of mitotic kinesin motor proteins in untransformed primary and tumor cells. We found striking differences for kinesin-like DNA binding protein (KID), involved in chromosome orientation during mitosis, and kinesin 5- Eg5, involved in bipolar spindle formation and microtubule sliding. Both proteins have previously been demonstrated to localize diffusely throughout the nucleus in some cell types. In this present study, we show that during interphase these motors each localize to the nucleolus of primary cells. However, in most tested cancer cell lines the nucleolar localization was not observed but the motors were instead diffusely localized in the nucleus. We are currently testing a model whereby mitotic motors are normally sequestered, and perhaps inactivated, in the nucleolus during interphase. Loss of the sequestration in cancer cells may promote a return to mitosis or be a consequence of abnormal cell cycle regulation in malignant cells.

2386

**Characterization of Mouse Kif26a, a Putative Vab-8 Orthologue**

E. Cid,[1] C. Zhan,[1] P. Höök,[2] R. Vallee,[2] M. Hatten[1]; [1]The Rockefeller University, New York, NY, [2]Pathology and Cell Biology, Columbia University, New York, NY

The aim of this study is to characterize KIF26A, a member of the N-11 kinesin family. Vab-8 is a *C. elegans* kinesin involved in neuroblast posterior migration and axon extension. Sequence comparisons between VAB-8 and mouse proteins resulted in the identification of KIF26A as a putative orthologue. It has a long N-terminal region before its kinesin motor domain, a neck region, and a short coiled coil segment at the carboxyl end. *Mm*KIF26A was cloned using RT-PCR and granule neuron phage library screening. Its expression pattern was determined by Northern Blot, RT-PCR and in situ hybridization. It is mainly restricted to the nervous system during embryogenesis. At E14.5 is localized in the cortex ventricular zone and cortical plate, in the spinal cord and sympathetic ganglia among other regions. In postnatal day 6 is present in the rostral migratory stream and olfactory bulb. In order to study the protein distribution a polyclonal antibody was raised against KIF26A. Immunolocalization in transfected COS7 cells showed a speckled pattern for KIF26A. The motor domain was overexpressed in bacteria as a GST fusion protein and its basal ATPase activity measured (188.1±14.6 nmol $P_i \cdot min^{-1} \cdot mg$ at 37°C). Two-hybrid analyses were also carried to assay a possible interaction between the kinase Unc51.1 and KIF26A. This interaction as has been described between their *C. elegans* counterparts UNC-51 and VAB-8. Correspondingly, the C-terminal half of the kinase interacts weakly with the central domain of KIF26A. In conclusion, KIF26A has been localized in the nervous system and is present in regions undergoing cell movements and axon extension. It also shares structural and functional characteristics with VAB-8 suggesting that it is a truly orthologue. More functional assay will clarify the role of KIF26A in neuroblast migration and axon extension in the mouse model.

2387

**Exposure of the Auxiliary Microtubule Binding Site in the Tail of Kinesin-1 Increases Processivity**

D. D. Hackney; Department of Biol. Sciences, Carnegie Mellon Univ., Pittsburgh, PA

Conventional kinesin-1 contains an auxiliary MT binding site (ABS) in the tail that is masked in folded full length DKH975, exposed in weakly folded DKH945 and DKH937 and lost in DKH894 (Nature Cell Biol. 2, 257 (2000)). Fusions between gelsolin and C-terminal truncations of kinesin have now been generated so that the influence of the ABS on processive movement can be monitored at the single molecule level by the method of Yajima et al (Curr.Biol. 12, 301 (2002)) that utilizes the ability of gelsolin to cap short fluorescently-labeled actin filaments. In agreement with the results of Yajima et al on short kinesin constructs lacking the ABS, DKH412GS moves in short runs along axonemes adsorbed to glass. Complexes of DKH941GS with F-actin, however, usually continue all the way to the end of an axoneme without dissociation. Thus exposure of the auxiliary MT binding site in DKH941GS significantly increases motile processivity. Furthermore, the sliding velocity of DKH941GS is not significantly decreased compared to DKH412GS and thus the ABS increases net MT affinity without binding so tightly as to impose a load that slows the rate of movement. DKH941GS also tends to remain attached on reaching the end of the axoneme and it accumulates in a bright fluorescence clump at the plus end. When the axonemes or microtubules are free in solution, they rapidly aggregate into asters with clumps of fluorescently-labeled DKH941GS at the foci. Thus even conventional kinesin-1 is capable of producing an accumulation of cargo molecules (F-actin in this model) at the plus ends of microtubules when its auxiliary MT binding site is exposed. The assistance of M. Parepally in cloning is gratefully acknowledged.

2388

**What Backstepping Tells Us About Kinesin Head Coordination**

N. R. Guydosh,[1] S. M. Block[2]; [1]Biophysics, Stanford University, Stanford, CA, [2]Biological Sciences and Applied Physics, Stanford University, Stanford, CA

The kinesin motor utilizes energy from ATP hydrolysis by both of its head domains to power processive motion over long distances. To maintain this processivity, the kinetic cycles of the two heads must be maintained out of phase (‘gated’) to ensure that at least one head remains tightly bound to the microtubule track at all times. Two broad classes of models explain this gating by invoking the effect of intermolecular strain that is generated at the point in the cycle when both heads are bound to the microtubule. In one class of models, rearward strain inhibits the front head until the back head catches up. In the other class, forward strain releases the back head before the front head proceeds prematurely. We tested these alternatives at the single-molecule level by probing the kinesin kinetic cycle using analogs of ATP and inorganic phosphate. Steps taken by native squid kinesin molecules bound to beads in an optical trap under force-clamped conditions were scored in the presence of ATP and beryllium fluoride ($BeF_x$). Binding of $BeF_x$ interrupts normal stepping by inducing long pauses. These events occur most often at high hindering loads or low

[ATP]. Our data are consistent with $BeF_x$ binding to the rear head before ATP can bind to the front head to trigger a normal forward step. During long pause events, we were able to resolve numerous short-lived backsteps of 8 nm. We found that normal forward stepping only resumed after a final, obligatory backstep, during which $BeF_x$ was presumably released from the front head. We conclude that the affinity of the front head for $BeF_x$ is reduced by strain while the back head remains unaffected, supporting models where strain leads to processivity by inhibiting the front head from productive nucleotide binding.

2389

**Force Generation by Ncd *in vitro* and in the Spindle**

S. A. Endow,[1] C. D. Yankel,[1] S. S. Rosenfeld[2]; [1]Cell Biology, Duke University, Durham, NC, [2]Neurology, Columbia University, New York City, NY

*Drosophila* Ncd is a nonprocessive minus-end Kinesin-14 spindle motor with a tail, coiled-coil stalk and C-terminal motor domain. We recently reported a new crystal structure of the dimeric motor showing a large displacement of the stalk compared to previous structures, indicating that the stalk can rotate and may act like a lever arm. FRET experiments now show a rapid movement of the stalk towards the microtubule after motor binding and away from the microtubule after hydrolysis, prior to motor release from the microtubule, identifying the steps at which the Ncd stalk moves and could amplify force. Analysis of live null-mutant oocytes and embryos expressing HeadlessNcdVenus and TaillessNcdVenus fluorescent proteins show that the Ncd tail is essential for binding to spindle microtubules and centrosomes. Fluorescence photobleaching experiments indicate that Ncd binding to meiotic spindles is primarily by the tail. The abnormal meiotic spindles of HeadlessNcd oocytes show that binding by both the Ncd head and tail to spindle microtubules is needed for spindle assembly. Mitotic spindles of HeadlessNcd embryos appear almost normal, suggesting that the role of Ncd in mitosis may be to crosslink microtubules to one another and attach chromosomes to the spindle, rather than to produce force for microtubule or chromosome movement.

2390

**High-Resolution Cryo-electron Microscopy on KIF1A-Microtubule Complex**

M. Kikkawa,[1] N. Hirokawa[2]; [1]Cell Biology, Univ. of Texas, Southwestern, Dallas, TX, [2]Cell Biology & Anatomy, Univ. of Tokyo, Graduate School of Medicine, Tokyo, Japan

Kinesin is a microtubule-based motor using ATP as the energy source. To understand how the chemical energy derived from ATP hydrolysis is converted to mechanical energy, we have been studying the structures of kinesin. Although previous X-ray crystallographic studies reveal two key conformations of the kinesin motor domain, the lack of high-resolution structures of the kinesin-microtubule complex prevents unambiguous correlation between a particular conformation and the nucleotide state. i.e. In the absence of microtubules, presumed ATP-like structures were observed even when ADP was bound, and a presumed ADP structure was observed with an ATP analogue. To clarify this and other issues, here, we used high-resolution cryo-electron microscopy and helical image analysis to obtain 10 Å resolution structures of the monomeric kinesin KIF1A-microtubule complex. By comparing the ADP- and the AMPPNP-state structures, we visualized most of the nucleotide-dependent conformational changes in the complex, including the switch II cluster, the neck linker, and the core rotation. These conformational changes are tightly coupled to the nucleotide-state. We also observed that the nucleotide-binding pocket is open in the ADP state, while the pocket is closed in the AMPPNP state. These structures are not observed in X-ray crystallographic structures, suggesting that the open/closed states of the nucleotide-binding pocket depend mainly on microtubules. Based on the new result, we propose a novel universal force generating mechanism, namely, “random diffusion & biased capture model”, which can explain asymmetric hand-over-hand movement of the dimeric kinesin as well as single-headed kinesin movement.

2391

**Stalk Region of Kinesin-related Protein Unc104 Has Moderate Ability to Form Coiled Coil Dimer**

Y. Shimizu,[1] H. Morii,[2] T. Shimizu,[2] F. Arisaka,[3] M. Tanokura[1]; [1]Grad. Sch. Agricult. Life Sci., Univ. Tokyo, Bunkyo-ku, Tokyo, Japan, [2]Inst. Biol. Resourc. Funct., Natl. Inst. Adv. Indust. Sci. Technol., Tsukuba, Japan, [3]Grad. Sch. Biosci. Biotechnol., Tokyo Inst. Technol., Yokohama, Japan

Unc104/KIF1A, a kinesin family member, is reported to be monomeric in solution, though its polypeptide has regions that potentially form coiled coils. It is also reported to carry synaptic vesicles along microtubules in nerve cells at a speed of 1.2 μm/s and that the movement is likely to be processive. The ‘hand-over-hand’ mechanism is proposed for processive movement of certain molecular motors, but for such a mechanism, dimerization of the protein would be necessary. To better understand the mechanism underlying Unc104/KIF1A’s motility, therefore, it is important to evaluate its dimerization ability. Thus, we have analyzed the stalk region of Unc104 from *C. elegans* by our program Amphisearch to confirm that several regions would have potential to form coiled coils. Based on this analysis, more than 20 peptides were synthesized. The CD measurements for those fragments indicated that the peptides having a common region (N358-K379) showed spectra characteristic to α-helix, while the peptides covering the other regions were likely to be random coils. Dimerization of the peptides described above by coiled coil formation was confirmed by analytical ultracentrifugation. From the concentration dependence of the CD spectra, the monomer-dimer dissociation constant Kd was estimated to be about 5 μM (slightly less than 1 mg prot./ml), considerably higher than that of the corresponding segment of human kinesin (62 nM). Though its dimerization ability is moderate, Unc104/KIF1A could nonetheless dimerize and, therefore, could move by the same mechanism as human kinesin when its concentration would get high. This further suggests that the motility of Unc104/KIF1A is controlled by the protein concentration.

2392

**Structure-Function Relationships of the Kinesin Superfamily Proteins (KIFs)**

H. Miki, Y. Okada, N. Hirokawa; Cell Biology and Anatomy, University of Tokyo, Bunkyo-ku, Japan

Kinesin superfamily proteins (KIFs) play fundamental roles in the intracellular transport system, which is essential for cellular function and morphology. KIFs are molecular motors which have been shown to transport organelles, protein complexes, and mRNAs to specific destinations along microtubules while hydrolyzing ATP for energy. KIFs also participate in chromosomal and spindle movements during mitosis and meiosis. It

is the first large protein family in mammals whose constituents have been completely identified and confirmed both *in silico* and *in vivo*. Numerous studies have revealed the structures and functions of individual family members, however the relationships between members or a perspective of the whole superfamily structure remain elusive. Here we present a comprehensive perspective based on a large, systematic phylogenetic analysis of the kinesin superfamily. All available sequences in public databases were analyzed to yield the most complete phylogenic kinesin tree thus far, comprised of 623 KIF sequences from all four biological kingdoms, animalia, plantae, fungi and protista. Over 98% of these KIFs are affiliated with 14 families. The clustering of KIFs to form groups that constitute subfamilies leading to the formation of families reveal the structural features that enlighten the cellular function of respective families. This comprehensive classification builds on the standardized nomenclature for kinesins and allows systematic analysis of the structural and functional relationships within the kinesin superfamily. Our full tree, viewable at our homepage (http://cb.m.u-tokyo.ac.jp/) is mainly comprised of uncharacterized KIFs, as the precise cellular function of relatively few have been elucidated. We hope this tree will aid in navigation through the unmapped areas of intracellular trafficking.

## Cilia & Flagella II (2393-2419)

2393

### Tissue Homeostasis is Regulated by the Primary Cilium in Fibroblasts

L. Schneider,[1] G. Pazour,[2] E. K. Hoffmann,[1] P. Satir,[3] S. T. Christensen[1]; [1]Department of Biochemistry, IMBP, The August Krogh Building, University of Copenhagen, Copenhagen, Denmark, [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worchester, Copenhagen, MA, [3]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY

We recently showed that the growth-arrest specific receptor tyrosine kinase, PDGFRα, is targeted to the primary cilium during growth arrest in NIH3T3 cells and primary cultures of mouse embryonic fibroblasts, MEFs [Schneider et al. (2005) Submitted]. PDGF-AA-induced activation of PDGFRαα in the cilium is followed by activation of the mitogenic Akt and the Mek1/2-Erk1/2 pathways and cell cycle entrance. Quiescent MEFs derived from *Tg737*$^{orpk}$ mutants fail to up-regulate PDGFRα, to form normal cilia and to activate Mek1/2-Erk1/2 and re-enter the cell cycle after stimulation with PDGF-AA. Here we investigated the role of the primary cilium in tissue homeostasis, in which MEFs were serum starved for 48 h to induce growth arrest. In wt cells, growth arrest was associated with activation of p53 by phosphorylation on serine in position 15. Wt cells remained viable and showed p53 phosphorylation for at least 144 h of serum starvation. In contrast, *Tg737*$^{orpk}$ mutants had a low level of phospho-p53 and after 96 h of serum starvation cells began to die by formation of apoptotic bodies and activation of caspase 3 such that all cells were dead after 144 h of starvation. Incubation with PDGF-AA or PDGF-BB could not compensate for this starvation-induced death. These results indicate that the primary cilium is required for activation of p53 to maintain cell quiescence and prevent cells from entering the apoptotic mode of programmed cell death. The ciliary signalling pathways regulating cell survival are presently unknown, although β1 integrin is shown to localize specifically to the primary cilium, indicative of the cilium signalling the interaction with the connective tissue and controlling tissue homeostasis. Thus the primary cilium in fibroblasts coordinates several fundamental cellular events in cell growth, survival, and potentially in migration supporting tissue homeostasis of connective tissues.

2394

### Mechanism of Nodal Flow: A Conserved Symmetry Breaking Event in Left-Right Axis Determination

Y. Okada, S. Takeda, Y. Tanaka, N. Hirokawa; Dept Cell Biol & Anatomy, Univ Tokyo, Grad Sch Med, Tokyo, Japan

The leftward flow in extra-embryonic fluid is critical for the initial determination of the left-right axis of mouse embryos. It is unclear if this is a conserved mechanism among other vertebrates and how the directionality of the flow arises from the motion of cilia. In this paper, we show that rabbit and medakafish embryos also exhibit a leftward fluid flow in their ventral nodes. In all cases, primary monocilia present a clockwise rotational-like motion. Observations of defective ciliary dynamics in mutant mouse embryos support the idea that the posterior tilt of the cilia during rotational-like beating can explain the leftward fluid flow. Moreover, we show that this leftward flow may produce asymmetric distribution of exogenously introduced proteins suggesting morphogen gradients as a subsequent mechanism of left-right axis determination. Finally, we experimentally and theoretically characterize under which conditions a morphogen gradient can arise from the flow.

2395

### Flagellar Motility as an Essential Function for Bloodstream African Trypanosomes

S. Griffiths, N. Portman, M. Shaw, S. Gordon, K. Gull; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Inducible RNAi is a useful tool for analysing gene function in the African trypanosome, *Trypanosoma brucei.* This organism is the etiological agent of African sleeping sickness, infecting the bloodstream of man and animals and being spread by the tsetse fly. The first RNAi motility mutants in trypanosomes (Bastin et al (1998) Nature **391**, 548) showed that one could produce viable but paralysed trypanosomes. Such a phenotype has subsequently been revealed for many flagellar RNAi mutants in trypanosomes. These phenotypes were obtained using strains of procyclic trypanosomes that represent the insect midgut form of the parasite. We have now made a direct comparison of a number of these RNAi mutants using the same constructs expressed in the bloodstream form parasites. In each case, expression of RNAi against the flagellar protein resulted in an impaired motility but viable phenotype in procyclic parasites, but an aberrant flagellum and death phenotype in bloodstream cells. Even though we employed RNAi knockdown of a diverse range of structural and regulatory proteins we found a similar aspect to the phenotype. Bloodstream cells produced a large flagellar pocket indicative of aberrant secretion/uptake traffic, flagellar and cell morphogenesis defects, and ultimate production of monstrous cells. Our results indicate the flagellum as having a critically important pathogenecity function in bloodstream trypanosomes and hence reveal motility as a therapeutic target.

2396

### Development of an Immortalized PCK Cholangiocyte Cell Line to Study ARPKD in vitro

T. Masyuk, M. Muff, B. Huang, P. Splinter, V. Torres, N. LaRusso; Internal Medicine, Mayo Clinic College of Medicine, Rochester, MN

ARPKD is caused by mutations in PKHD1, the gene that encodes the protein, fibrocystin (FC). The formation of liver cysts in the PCK rat (a rodent model of ARPKD resulting from a mutation in Pkhd1) is associated with morphological abnormalities of cholangiocyte cilia (they become

shorter and malformed) and alterations in the FC intracellular location (it disappears from cilia but is expressed in the cytoplasm; Gastroenterology 2003, 125:1303). However, the relationships among mutations in FC, malformed cholangiocyte cilia, FC topographical alterations, and hepatic cystogenesis remain unclear. To continue to clarify these relationships, we developed a spontaneously immortalized PCK-derived cholangiocyte cell line (PCK-CCL). These cells were maintained in culture for one year over twenty passages. They continue to express cholangiocyte markers cytokeratins 7 and 19, and GGT, but were essentially devoid of desmin and vimentin, non-epithelial cell markers. The cells showed typical cholangiocyte morphology: large, notched nuclei, numerous microvilli on the apical surface, and tight junctions. Compared to a normal rat cholangiocyte cell line (NRC), the PCK-CCL exhibits higher electrical resistance (2200 ohmscm2 vs 1500 ohmscm2), and possesses short, malformed cilia (1.29±0.08um vs 7.64±1.7um), abnormalities similar to what we observed in PCK cholangiocytes in vivo. Moreover, the PCK-CCL forms spherical cysts in a 3D collagen matrix and the number of cystic structures, rate of cyst formation and the cystic area are significantly greater in the PCK-CCL than in NRC. Finally, the PCK-CCL expresses FC in the cytoplasm and plasma membrane and not in the cilia. In conclusion, we have developed and characterized a non-malignant, spontaneously immortalized cholangiocyte cell line from the PCK rat to allow further studies on the mechanisms involved in hepatic cyst formation, expansion and progression and to test treatment approaches for inherited cystic liver disease.

2397

### Primary Cilia in Fibrosis Associated with Two Models of Polycystic Kidney Disease

S. R. McGlashan,[1] C. A. Poole,[2] C. Stayner,[3] A. C. Johnstone,[4] M. R. Eccles,[3] C. G. Jensen[1]; [1]Dept. of Anatomy with Radiology, University of Auckland, Auckland, New Zealand, [2]Section of Orthopaedic Surgery, Dept. of Medical and Surgical Sciences, University of Otago, Dunedin, New Zealand, [3]Dept. of Pathology, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand, [4]Pathobiology Section, Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand

The two characteristics of polycystic kidney disease (PKD) are the development of cysts and fibrosis. Fibrosis, a common cause of renal failure, results from excessive interstitial fibroblast proliferation and extracellular matrix production. Although previous studies of PKD have concentrated on primary cilia defects of cyst epithelia, we hypothesize that abnormalities in fibroblast primary cilia lead to fibrosis. Our study aimed to characterize interstitial fibrosis and fibroblast primary cilia expression in two separate models of PKD - an autosomal dominant PKD mouse ($Pkd1^{del34/del34}$) and a recently-described) autosomal recessive PKD in sheep (Johnstone *et al*., 2005, NZVJ). Kidneys from E18.5 $Pkd1^{(del34/del34)}$ and wild-type mice, and from newborn wild-type and mutant lambs were fixed, wax-embedded and sections were stained using H&E or Masson's Trichrome. Indicators of fibrosis were the mean percentage area and cell count of the interstitium. Primary cilia were labeled using an acteylated α-tubulin antibody. Moderate-to-severe fibrosis surrounded cysts in both the mutant kidneys with an increase in fibroblast numbers. Primary cilia were present on tubular epithelia and interstitial cells in both wild-type mouse and lamb kidneys. In Pkd1 kidneys, primary cilia were associated with both unaffected epithelia and cyst epithelia and there was no difference in length between epithelial cilia of the cysts and those of wild-type tubules. However, only a small proportion of Pkd1 fibroblasts expressed primary cilia. In contrast, in the lamb, the majority of cyst epithelia did not express primary cilia and interstitial cells expressed only stunted cilia. This is the first study to describe fibrosis in two models of cilia-related PKD. The presence of primary cilia in cyst epithelia confirms reports that the Pkd1 mutation results in a functional ciliary defect; in the lamb, the absence of primary cilia suggests a structural abnormality.

2398

### The Cytoplasmic Tail of Fibrocystin/Polyduction Contains A Ciliary Targeting Sequence

L. Li, J. Follit, E. Luna, G. Pazour; Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

The human autosomal recessive polycystic kidney disease gene (PKHD1) encodes an ~450 kD protein called fibrocystin or polyductin that is predicted to be almost entirely extracellular with a short 192 amino acid cytoplasmic tail (Ward et al., Nat Genet. 30(3):259-69; Onuchic et al., Am J Hum Genet. 70(5):1305-17). Fibrocystin localizes to cilia and centrosomes in cultured mammalian cells (Ward et al., Hum. Mol. Genet. 12(20):2703-10; Wang et al., JASN 15(3):592-602) and it is likely that the cytoplasmic tail plays a role in targeting to these organelles. To test this idea, the cytoplasmic tail of mouse fibrocystin was fused to GFP and expressed in kidney cells. In non-ciliated cells, the tagged protein localizes around centrosomes; in ciliated cells the protein is found predominately in the cilia. Dissection of the cytoplasmic tail sequence identified an 18 amino acid peptide that is sufficient to target GFP to cilia. This sequence is highly conserved in vertebrates and contains cysteine residues that are likely to be palmitoylated. Mutation of these cysteines to alanine or treatment of the cells with 2-bromopalmitate, an inhibitor of palmitoylation, abolish the ability of this peptide to target to the cilium. Certain point mutations within the ciliary targeting sequence prevent ciliary targeting but instead cause the protein to accumulate in small punctate spots on the plasma membrane. These spots also label with cholera toxin B, which binds GM1 and is a marker for lipid rafts. Palmitoylation can target proteins to lipid rafts, thus we propose that the ciliary targeting event is a two step process whereby the protein is first targeted to GM1-rich regions of the plasma membrane and then to the ciliary membrane.

2399

### A Homologue of Polycystic Kidney Disease Protein Polycystin-2 is Present in Chlamydomonas Flagellar Membranes

K. Huang,[1] G. J. Pazour,[2] G. B. Witman,[3] M. Hippler,[4] J. L. Rosenbaum[1]; [1]MCDB Department, Yale University, New Haven, CT, [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, [3]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA, [4]Department of Biology, University of Pennsylvania, Philadelphia, PA

The proteins encoded by most polycystic kidney disease (PKD) genes are found, in part, on cilia, and defects in ciliary assembly in vertebrates cause PKD. These observations led to the "ciliary hypothesis of PKD". Several PKD gene homologues are found in the Chlamydomonas flagellar proteomic database, including polycystin-2 (PKD2) (C_590099), polyductin/fibrocystin (C_420012) and qilin (C_10017). We cloned the Chlamydomonas PKD2 gene; it has 12 exons that encode a protein of 180 kD, which contains 4 putative leucine zipper pattern domains, an EF-hand domain, and six transmembrane domains, which are characteristic of PKD2 family members. Using antibodies generated against either the first extracellular loop or a small peptide from the C-terminus, two polypeptides (180 kD and 120 kD) were detected in the cell body by immunoblotting. The smaller of these was the predominant flagellar form, and was enriched in a flagella membrane fraction. PKD2 could be visualized in the flagella by immunofluorescence microscopy. When the temperature-sensitive mutant *fla10*, which harbors a defective subunit of

the anterograde intraflagellar transport (IFT) motor Kinesin-2, was shifted to the restrictive temperature, the amount of PKD2 in the flagella increased. These data suggest that IFT is involved in the turnover of PKD2 in flagella. A PKD2-GFP fusion construct and an RNA interference construct have been made and transformed into Chlamydomonas. We are screening and analyzing the transformants to determine the dynamics and function of Chlamydomonas PKD2 in the flagellum. Supported by NIH grants GM14642 to JLR and GM30626 to GBW.

2400

**Cell Cycle Regulation of Primary Cilium Biogenesis in Mammalian Cells**

C. T. Anderson, T. Stearns; Biological Sciences, Stanford University, Stanford, CA

In mammalian cells, primary cilia normally grow from mother centrioles during early G1 phase, but the regulatory mechanisms by which cells control primary cilium biogenesis are poorly understood. We tested whether cells require specific cell cycle events and regulators in order to generate primary cilia. Cells arrested in mitosis by expression of non-degradable cyclin B do not generate primary cilia, while cells that are $p53^{-/-}$ or $pRb^{-/-}/p107^{-/-}/p130^{-/-}$, cells that fail cytokinesis after treatment with the actin inhibitor cytochalasin B or the myosin II inhibitor blebbistatin, cells arrested in S phase by hydroxyurea or aphidicolin treatment, and cells arrested in G2 by etoposide treatment are able to generate new primary cilia. Thus, primary cilium biogenesis requires cyclin B degradation, but not completion of cytokinesis or the pRb family or p53 regulatory proteins. In addition, primary cilium biogenesis can occur during S or G2 arrest, indicating that the maturation process by which centrioles become competent to form primary cilia, and the growth of primary cilia themselves, may be independent of normal cell cycle progression.

2401

**Testing Models of Flagellar Length Control**

K. A. Wemmer, W. F. Marshall; Biochemistry & Biophysics, University of California, San Francisco, San Francisco, CA

Cilia and flagella are organelles that serve a diverse set of functions in a wide range of organisms. Despite the importance of these structures, the mechanisms driving assembly of the axoneme and maintenance of its length are poorly understood. It is possible to study these mechanisms in the flagellated unicellular green alga *Chlamydomonas reinhardtii*, as cilia and flagella have a high degree of structural conservation across species. Here, we focus on how length is tightly and precisely controlled. It is known that the distal tips of flagella are unstable and undergoing continuous turnover, thus the precise length of the flagella must be maintained by balancing assembly and disassembly at the tip of each flagellum. Several models have been proposed to explain the mechanism responsible for this balance. We would like to test these models by examining flagellar length mutants using a combination of genetics and microscopy to test predictions derived from these models of length control.

2402

**Phosphoinositides are Required for Spermatid Elongation in *Drosophila***

H. Wei; Developmental Biology, The Hospital for Sick Children, Toronto, ON, Canada

During sperm tail formation, cellular membranes undergo dramatic remodeling, concomitant with assembly of a microtubule-based axoneme. The mechanisms that coordinate these membrane and cytoskeletal changes in *Drosophila* spermatogenesis are unknown, although genes required for spermatid elongation have been identified. Two such genes, *syx5* and *fws*, encode proteins involved in membrane trafficking, suggesting that an intact secretory pathway is critical for membrane remodeling during sperm tail formation. Phosphatidylinositol phospholipids (PIPs) modulate membrane trafficking, actin cytoskeletal organization and signal transduction. To deplete PIPs and determine if these key regulatory phospholipids affect sperm tail formation, we expressed the *Salmonella* PIP phosphatase, SigD, in developing male germ cells. SigD depleted $PI(4,5)P_2$ from the plasma membrane and caused profound defects in spermatid elongation. These defects were largely rescued by co-expression of PIP 5-kinase, confirming that PIPs are needed for normal spermatid differentiation. Septins typically localize to stable intercellular bridges and to punctate structures that lie along the length of elongating axonemes. In contrast, SigD-expressing testes exhibit abnormal septin filaments and few, if any, axonemes. We determined that *Drosophila* septins, like their mammalian homologs, bind PIPs *in vitro*, suggesting PIPs may modulate septin polymerization. In addition, centrosomin and γ-tubulin, basal body proteins that are not thought to bind PIPs directly, were mislocalized in postmeiotic spermatids from PIP-depleted testes. We speculate that PIPs promote sperm tail formation by influencing membrane trafficking, septin organization, as well as localization and/or activation of basal body proteins required for axoneme assembly.

2403

**PDGF-AA Induced Activation of the Mitogenic Erk1/2-Mek1/2 Pathway Localizes to the Primary Cilium and the Ciliary Basal Body in NIH3T3 Fibroblasts**

C. A. Clement,[1] L. Schneider,[1] S. C. Teilmann,[1] P. Satir,[2] S. T. Christensen[1]; [1]Department of Biochemistry, IMBP, The August Krogh Building, University of Copenhagen, Copenhagen, Denmark, [2]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY

Primary cilia are present in most quiescent tissue cells in our body as solitary organelles that emerge from the mother centriole that has become a basal body. Our research focuses on the function of the primary cilium in connective tissues in tissue homeostasis, growth control and migration in wound healing. We previously showed that PDGFRαα-mediated transition from growth arrest to cell cycle entrance is initiated through activation of the receptor homodimer in the primary cilium of quiescent fibroblasts followed by activation of the mitogenic Mek1/2-Erk1/2 pathways [Schneider et al. (2005) Curr. Biol., Submitted]. Here we investigated the subcellular position of the signal transduction cascades initiated by ligand-dependent activation of ciliary PDGFRαα through phosphorylation of the receptor docking site for Shp/Grb2 that leads to activation of Mek1/2 through the Ras-Raf pathway in NIH3T3 fibroblasts. Co-localization studies with anti-acetylated α-tubulin and anti-pericentrin show that PDGF-AA increases the level of phospho-Mek1/2 (c-Raf-dependent Mek1/2 phosphorylation in the activation loop on serines in positions 217 and 221 that activates Erk1/2) along the cilium and at the base of the cilium, i.e. to the mother centriole, and not to the daughter centriole. Further, Mek1 is phosphorylated in the cilium on serine in position 298, which is executed by p21-activated protein kinase, PAK, and which is a convergence point for integrating growth factor signaling via the MAPK pathway. We also demonstrate feed back inhibition of Mek2 by Erk-mediated phosphorylation of threonine on position 394 in the primary cilium, supporting the conclusion that Raf-dependent activation of Mek1/2 is assisted by PAK in the cilium, and that the primary cilium contains the signalling machinery from PDGFRαα activation to Mek1/2 through the

Ras-Raf pathway.

2404

**Activated Disassembly of the Axoneme and Increased Trafficking of IFT Particles during Shortening of Eukaryotic Flagella**

J. Pan, W. J. Snell; Cell Biology, UT Southwestern Medical Center, Dallas, TX

Cells possess mechanisms not only to assemble and maintain cilia/flagella but also to shorten them. We are using *Chlamydomonas* to study the mechanisms that regulate the flagellar shortening that is induced by environmental and developmental cues. Previously, we showed that the aurora protein kinase CALK plays a central role in the regulation of flagellar shortening. When cells receive cues to shorten, CALK is phosphorylated and its protein kinase activity is stimulated. Moreover, RNAi knockdown of CALK inhibits shortening. Here we report on studies of the cellular mechanisms of flagellar shortening. Phosphopeptide analysis of CALK shows that triggers for shortening lead to phosphorylation of CALK on several sites, suggesting that it is regulated by more than one protein kinase. In studies on events downstream of CALK, we found that the flagellar shortening pathway is independent of the Lf4 protein, which is essential for the transient shortening that enforces flagellar length. Studies on the rates of shortening in wild type cells and mutants with a conditional lesion in the anterograde intraflagellar transport (IFT) motor, kinesin 2, immunoblot analysis of the amounts of IFT particle proteins and motors in steady state and shortening flagella, and analysis of anterograde and retrograde cargo in steady state and shortening flagella have led to the following model for shortening. When triggered to shorten their flagella, cells stimulate disassembly of the axoneme at the tip over the basal rate, they increase by 4-fold trafficking of IFT particle proteins in the flagella, and the newly entering IFT particles are empty of cargo. Thus, during flagellar shortening cells increase the rate of entry of empty IFT particles into the flagella to pick up the disassembled flagellar components generated by active disassembly. Supported by NIH.

2405

**Adenylate Kinases in the Eukaryotic Flagellum: Protein Targeting, Energy Metabolism, and a Novel Related Gene Family Required for Motility**

E. Barnwell, K. Gull, M. Ginger; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Three novel adenylate kinases in the flagellar proteome of the protozoan parasite *Trypanosoma brucei* (causal agent of African sleeping sickness) provide prime examples of metabolic enzymes which are anchored within the trypanosome's flagellar architecture. These enzymes differ from ubiquitous cytoplasmic and mitochondrial adenylate kinase isoforms by the presence of N-terminal extensions of approximately fifty amino acids, which are both necessary and sufficient for flagellar targeting. Intriguingly, using a bioinformatics approach, these flagellar targeting sequences are sufficient to identify unusual tandem adenylate kinases in the genomes of other eukaryotic flagellates, including nematodes, humans, and the ciliate *Tetrahymena thermophila.* The *T. brucei* flagellar proteome also contains two examples of larger proteins (m.w. >120kDa) that contain the pfam adenylate kinase domain. However, the primary sequence homology with adenylate kinase is disrupted in several places by insertions of unrelated amino acid sequences. We refer to these novel polypeptides as adenylate kinase-related proteins, and find examples of this "split adenylate kinase" theme in all eukaryotic flagellates for which complete or extensive genome coverage is available. Significantly, we do not find examples of unusual adenylate kinases or adenylate kinase-related proteins in the genomes of eukaryotes which do not build a cilium or flagellum. We show by epitope-tagging that both trypanosome adenylate kinase-related proteins are present in the axoneme. We speculate that our characterisation of the *T. brucei* flagellar adenylate kinases provides evidence for a flagellar energy generating capacity that is determined by the specific niche environment occupied by trypanosome parasites. RNA interference-mediated gene silencing demonstrates that an absence of the adenylate kinase-related proteins results in motility defects in cultured parasites.

2406

**The *Chlamydomonas* Flagellar Axoneme Contains a cGMP-dependent Protein Kinase (PKG) that Regulates Axonemal Motility**

B. Verhalen, A. R. Gaillard; Biological Sciences, Sam Houston State University, Huntsville, TX

Studies of cilia and flagella in various systems have revealed a role for cGMP and cGMP-dependent kinase (PKG) in the regulation of axonemal motility. To determine whether the *Chlamydomonas* axoneme contains a PKG that is involved in the regulation of axonemal motility, we performed both pharmacological and biochemical experiments using *Chlamydomonas* axonemes. We first performed studies of reactivated cell motility using demembranated cell models. These studies revealed that the percentage of motile cells decreases when reactivation of wild-type cells is performed in the presence of the PKG inhibitor Rp-8-pCPT-cGMPS, indicating that PKG is a regulator of axonemal motility. We then performed Western blot analysis of axonemal protein from wild-type cells using a peptide antibody to a putative *Chlamydomonas* PKG, revealing the presence of two protein bands of approximately 110 and 120 kD. The sizes of these protein bands are consistent with the predicted size of 105 kD for the product of genie 214.2, a putative *Chlamydomonas* PKG gene. Studies of axonemal protein samples from various structural mutants have demonstrated that the 110 and 120 kD proteins are not exclusively localized to the radial spokes, central pair apparatus, dynein regulatory complex, I1 inner dynein arm, or the outer dynein arms. The 110 and 120 kD proteins are extractable with 0.6 M NaCl, and sucrose gradient fractionation of 0.6 M NaCl extracts has shown that the 110 kD protein sediments at approximately 10S, while the 120 kD protein sediments at around 12S. These results suggest that *Chlamydomonas* axonemal PKG may exist as part of one or more protein complexes.

2407

**Intracellular Ca2+ and cAMP Signaling Pathways Are Involved in Mechanosensory Function of Primary Cilia in Biliary Epithelia**

A. I. Masyuk, T. V. Masyuk, P. L. Splinter, B. Q. Huang, A. J. Stroope, N. F. LaRusso; Internal Medicine, Mayo Clinic College of Medicine, Rochester, MN

Cholangiocytes, the epithelial cells lining intrahepatic bile ducts, contain primary cilia extending into the ductal lumen. The functions of cholangiocyte cilia are unknown. Studies on primary cilia in renal epithelia suggest that these organelles function as mechanosensors. In present study, we tested the hypothesis that cholangiocyte cilia are mechanosensory organelles that detect and transmit luminal bile flow stimuli into intracellular signaling responses. Scanning and transmission electron and immunofluorescent confocal microscopy of rat isolated intrahepatic bile duct units (IBDUs) were employed to assess cholangiocyte cilia. Changes in cholangiocyte [Ca2+]i and [cAMP]i in response to luminal perfusate flow in microperfused IBDUs were measured by epifluorescence microscopy and a fluorescence assay, respectively. We found that each

cholangiocyte in IBDUs contains a primary cilium on its apical plasma membrane. Cholangiocyte cilia express a mechanoreceptor, polycystin-1 (PC-1), a Ca2+ channel, polycystin-2 (PC-2) and the Ca2+-inhibitable adenylyl cyclase isoform 6 (AC6). Perfusion of IBDUs at flow rates of sufficient magnitude to bend cilia resulted in a 40 % increase in [Ca2+]i and a four-fold decrease of forskolin-stimulated [cAMP]i levels. The flow-induced changes in [Ca2+]i and [cAMP]i were significantly reduced or abolished when cholangiocyte cilia were removed by chloral hydrate or when PC-1, PC-2 and AC6 were individually down-regulated by siRNAs. In conclusion, cholangiocyte cilia contain PC-1, PC-2 and AC6 through which luminal flow stimuli affect both the [Ca2+]i and cAMP signaling pathways, two pathways known to be involved in regulation of ductal bile secretion.

2408

**Evidence That $Ca^{2+}$ Selectively Affects Dyneins on Certain Microtubule Pairs in the Mouse Sperm Axoneme**

K. C. Ponichter, K. A. Lesich, C. B. Lindemann; Biological Sciences, Oakland University, Rochester, MI

Mouse sperm were induced to undergo sliding disintegration of the axoneme by a two step method. First, epididymal sperm were detergent extracted with 0.1% Triton X-100 in a potassium glutamate based reactivation buffer in the presence of 0.3mM Mg-ATP at pH 7.8 as described in Lindemann and Goltz, 1988 (Cell Motil. Cytoskeleton, 10:420-431). A high percentage (~90%) of sperm exhibited a coiled “curlicue” shape if 1mM $Ca^{2+}$ was also added to the mixture. Second, the cells were stripped of their mitochondrial sheath by transferring 0.5 ml of the reactivated cells to 4.5 ml of an extraction buffer that contained 0.1 M potassium glutamate, 2mM DTT, 0.02M Tris-HCl, 5mM $MgCl_2$, 0.5mM EGTA, 1mMATP and 0.5% Triton X-100 at pH 9.5 and incubating at 34ºC for 5 min. After incubation in the extraction buffer, phase microscopy revealed disintegrated axonemes with extruded outer dense fibers and microtubules. The polarity of the disintegration patterns was analyzed with respect to the reference provided by the pointed asymmetric sperm head. In the absence of $Ca^{2+}$ most cells had emergent fibers that came from the side of the axoneme opposite to the head point (47% vs. 14%). This ratio was shifted to almost equal numbers in the samples treated with $Ca^{2+}$ (28% vs. 27%). There were also significantly more cells showing fibers ejected from both sides of the axoneme in the $Ca^{2+}$ treated samples (6% control; 12% with $Ca^{2+}$; $p < 0.05$). These results suggest that the dyneins on doublets #1 and #2 are more likely to actively initiate sliding in the presence of $Ca^{2+}$.
Supported by NSF grant MCB-0110024.

2409

**The Evolution of Flagellar Length Control: Novel IFT Kinesin Function in the Basal Eukaryote Giardia Intestinalis**

S. C. Dawson, M. S. Sagolla, W. Z. Cande; MCB, UC-Berkeley, Berkeley, CA

*Giardia* intestinalis belongs to the most basal group of eukaryotes, and is a model for cytoskeletal evolution. Control of flagellar length is an important question in biology, as links exist between proper flagellar function and human disease. Intraflagellar transport (IFT) is the process responsible for flagellar assembly and maintenance in *Chlamydomonas*, yet this mechanism has not been studied in *Giardia*. Many components of anterograde and retrograde IFT, and two kinesin-2 homologs, are present in the *Giardia* genome. We are unable to find cytoplasmic dynein, a component essential for retrograde IFT in other eukaryotes. Using GFP-tagging, we show that conserved IFT proteins (kinesin-2, EB1, IFT-81) show conserved localization to extracellular regions, docking sites, and flagellar tips of all axonemes. Further, the overexpression of a dominant negative kinesin-2 inhibits the growth of only external axonemes. Interestingly, the MT depolymerizing kinesin-13 homolog has a novel localization to flagellar tips, and flagellar growth appears to be affected by the overexpression of a dominant negative. Unique intracellular regions characterize the four pairs of giardial axonemes. Several novel giardial kinesins also localize only to intracellular axonemes. Finally, unlike *Chlamydomonas*, flagellar length in *Giardia* is affected by the microtubule-stablilizing drug taxol and destablizing drug nocodazole. Anterior and ventral flagellar pairs have a greater sensitivity to nocodazole than other flagellar pairs. These data support the balance point model for regulation of flagellar length and suggest a novel mechanism of regulation for the internal versus external axonemes. IFT is largely a conserved process in *Giardia*, despite the novel role for a depolymerizing kinesin in flagellar disassembly. Although we propose an ancient origin of anterograde IFT (likely present in ancestral eukaryotes), we speculate that *Giardia* diverged before the evolution of “conventional” retrograde IFT.

2410

**Intraflagellar Transport Particles Participate Directly in Signal Transduction During Cilium-generated Signaling in *Chlamydomonas***

Q. Wang, W. Snell; Cell Biology, UT Southwestern Medical Center, Dallas, TX

Cilia are widely used as sensory transducers in both unicellular and multicellular organisms. Several previously poorly understood human disorders are now known to be cilia-related disorders. The most common inherited disease in humans, polycystic kidney disease, is due to a failure of cilium-generated signaling in kidney epithelial cells. And Bardet-Biedl Syndrome, characterized by obesity, mental retardation, and diabetes, is due to defects in cilia genes. The intraflagellar transport (IFT) machinery, powered by anterograde motor kinesin-II and retrograde motor cytoplasmic Dynein 1b transports proteins between the cell body and the cilium. Here we show that IFT is essential not only for cilia/flagella assembly and maintenance, but also for modulation of cilium-based sensory processes. In studies on the biflagellate alga, *Chlamydomonas* we find that IFT is essential for maintaining a high concentration of a soluble signaling protein, cGMP-dependent protein kinase (PKG), in the flagella. During fertilization, flagellar adhesion between gametes of opposite sex activates a signal transduction pathway leading to gamete activation and cell fusion. RNA interference and pharmacological experiments indicate that the PKG is involved in this pathway and is essential for fertilization. Analysis using immunopurification and mass spectrometry shows that the PKG is activated and tyrosine phosphorylated by a protein tyrosine kinase, which is activated by flagellar adhesion. Moreover, biochemical fractionation of flagella isolated from non-adhering and adhering gametes indicates that adhesion causes the PKG to become part of a multiprotein signaling complex that includes IFT particles; generation of signaling requires kinesin-II. Thus, in addition to its role in transporting flagellar structural components between the flagella and the cell body, IFT plays a direct role in cilium-generated signaling. Supported by NIH GM25661

2411

**Signaling in *Tetrahymena* Cilia: Evolution of Ciliary Signaling for Mammalian Growth Control Regulation**

C. F. Guerra,[1] A. J. Bell,[1] A. Awan,[1] S. T. Christensen,[2] P. Satir[1]; [1]Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]Institute of Molecular Biology, University of Copenhagen, Copenhagen, Denmark

Environmental sensing and cell signaling by ciliary membrane proteins are especially significant for single celled ciliated organisms, e.g. *Tetrahymena*, not only for rapid behavioral responses, but also for longer term responses leading to fission or mating. Recently we have reported on a ciliary membrane bound protein with a proposed receptor tyrosine kinase function (TtPTK1, Christensen et al ,2003), which has sequence motifs homologous to insulin-like growth factor (IGF) receptors. We have searched the *Tetrahymena* database for other molecules to complete this growth control signaling pathway. SH2-domain containing proteins, tyrosine phosphatases and MAP kinases are all present. *Tetrahymena* database protein #16928 is homologous to *C.elegans* EMB-5, an SH2 domain protein, which interacts with a transmembrane receptor. Rad 51 has been identified in *Tetrahymena* (Campbell and Romero,1998) and reportedly interacts with IRS-1 (Trojanek et al, 2003). We have designed probes based on database sequences for these and similar potential ciliary signaling proteins in *Tetrahymena* to test for mRNA upregulation during ciliogenesis. Upregulation is taken to be a sign that the protein is expressed in connection with the cilium, and could be part of the signaling system. TtPtk1, Gef1, Rad51 and PI3K are upregulated by 2.87, 4.37, 8.16 and 15.47 fold respectively, while control proteins are not upregulated**.** These findings are consistent with other reports of ciliary proteomics (Pazour et al 2005, Smith et al 2005) identifying several signaling proteins within the cilium. We conclude that growth factor signaling pathways starting in the ciliary membrane, which influence and directly control cell survival, chemotaxis and division, are present in *Tetrahymena*, which suggests that such pathways arise early in eukaryotic cell evolution and are retained.

2412

### Protein Tyrosine Kinase Activation and Cilium-Generated Signaling in *Chlamydomonas*

Q. Wang, W. Snell; Cell Biology, UT Southwestern Medical Center, Dallas, TX

Fertilization in the biflagellated green alga *Chlamydomonas* is initiated by flagellar adhesion between mt+ and mt- gametes. Recently, we showed that activation of a flagellar protein tyrosine kinase (PTK) is an early event during this cilium-generated signaling process. In order to identify protein tyrosine kinases in *Chlamydomonas* , we performed bioinformatics screening of the *Chlamydomonas* JGI v.2 genome database. We identified three predicted proteins as putative PTK's, which represent less than 0.15% of the total predicted proteins in *Chlamydomonas* (~20,000). This percentage is much less than that in Metazoan (~2%), but is larger than that in *Arabidopsis* and rice, which contain no predicted PTKs. It is possible that the PTK's are related to cilia, since higher plants do not contain these organelles. According to Clustal W analysis, the *Chlamydomonas* PTK's have about 30% identity in the catalytic domains with known protein tyrosine kinases, including high similarity in the PTK active site (VIb) signature (prosite number PS00109). However, all three lack the tyrosine residue around the DFG motif, suggesting that they may not be regulated by tyrosine phosphorylation. We cloned two of the putative PTK genes and have generated anti-peptide antibodies. Experiments are in progress to identify and characterize the proteins. Supported by NIH GM25661.

2413

### Another Chlamydomonas NIMA-related Kinase with a Flagellar Function

J. D. K. Parker, L. M. Quarmby; MBB, Simon Fraser University, Burnaby, BC, Canada

NIMA-related kinases (Neks) are best known in mammals as cell cycle regulators, but the Chlamydomonas Neks Cnk2p and Fa2p have flagellar functions in addition to cell cycle roles. Over- and under-expression of Cnk2p leads to defects in flagellar length (Bradley & Quarmby, 2005), while fa2 mutants have defects in flagellar disassembly (Mahjoub et al., 2002; Parker & Quarmby, 2003). Additionally, studies in Tetrahymena have implicated the Nek family as regulators of flagellar length (Wloga et al., 2004). The genes for several Chlamydomonas long flagella (lf) mutants have been cloned, but the identities of the genes mutated in short flagella (shf) mutants have not yet been reported. As the shf1 mutation maps near to the CNK3 gene, we are testing the hypothesis that CNK3 carries the shf1mutation. Expression of the wild-type CNK3 cDNA rescues shf1 mutants, while stable CNK3[RNAi] lines have short flagella. At this writing, we are sequencing the CNK3 gene from shf1 mutants. Cnk3p is the closest Chlamydomonas homologue of mouse Nek1 (54% identity in the kinase domains). Because Nek1 is the causative gene in the kat murine model of the ciliopathy autosomal recessive polycystic kidney disease, we thus speculate that Nek1 may have a ciliary function.

2414

### Ciliary Waveform Analysis of Human Airway Epithelium Using High Speed Differential Interference Contrast Video Microscopy

P. R. Sears, C. Davis; Cystic Fibrosis Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

The shape of a cilium depends on the activation of dynein motors and viscous drag. Our preparation allows precise measurements of this shape and inferences of activation. A 1 cm wide culture is wrapped around the edge of a square 18x18 mm #1 coverglass and placed on a 55x25 mm #1 coverglass. A second 18x18 coverglass is placed on top and overhangs the cells by 3 mm. Silicone grease secures the assembly and circumscribes the medium (DMEM-F12). The cells are observed using a 40x or 60x oil objective (NA $\geq$ 1.3) and up to 3x post-objective magnification. A Megaplus ES-310T camera is used at 125-250 frames per second. The cilium was divided into two 3.5 µm regions. The base region assumes maximum positive and negative curvatures at the beginning of the reverse and forward strokes and the two motions are identical relative to their respective starting positions and durations. For the tip region, the magnitude of the curvature is greater during the reverse stroke than the forward stroke. The beginning of the forward stroke is marked by a highly curved configuration while the beginning of the reverse stroke has low curvature. The base region curvatures and standard errors corresponding to the beginning of the forward and reverse strokes are ($\mu m^{-1}$) -0.52 ± 0.06 and 0.55 ± 0.04, respectively. For the tip region they are 0.85 ± 0.06 and 0.14 ± 0.1. n = 3. The base region may not be activated differentially during the forward and reverse strokes except for the subset of motors activated. The tip region motion can be explained by either differential activation or asymmetrical structures.

2415

### Theoretical Analysis of Balance-point Model for Flagellar Length Control System

W. Marshall; Biochemistry, UCSF, San Francisco, CA

Flagella are an excellent model system for studying the mechanism of organelle size control, because their size can be specified by a single number - the flagellar lenght. We have previously presented a balance-point model for flagellar length control, based on our analysis of flagellar microtubule turnover and intraflagellar transport. Here we analyze the behavior of this model as a function of individual parameters, and indicate how flagellar length distribution are predicted to change in mutants affecting specific aspects of turnover and IFT. We investigate the system

requirements for this model to work in order to formulate the most general possible version of the model. We explore the noise-resistance of the proposed length control system and the ability of the system to equalize flagellar lengths in response to transient alterations in length. We also indicate how similar models can account for size control in microvilli and other organelles.

2416

**Estimation of Ciliary Waveform Changes in Axonemal Dynein Mutants of Tetrahymena by Ciliary Beat Angle Quantitations**

D. Y. Kim,[1] D. G. Pennock,[2] T. M. Hennessey[1]; [1]Department of Biological Sciences, University at Buffalo, Amherst, NY, [2]Department of Zoology, Miami University, Oxford, OH

Swimming speed in *Paramecium* and *Tetrahymena* is governed by both the ciliary beat frequency and ciliary waveform of somatic cilia. It has been suggested that outer arm dyneins (OADs) of the ciliary axoneme are necessary for beat frequency changes and that inner arm dyneins (IADs) determine waveform. Ciliary waveform affects the direction of ciliary beating and the direction of the powerstroke. In *Paramecium*, it has been shown that depolarizing stimuli cause an increase in intracellular Ca++, a counter-clockwise shift of the powerstroke and a wider helical path due to the cilia beating more across the body than anterior-posterior. This decreases forward motion and swim speed. Threshold depolarizations produce Ca++-based action potentials, ciliary reversal and backward swimming. Detergent-permeabilized, reactivated *Paramecium* "models" have been used to show that such ciliary waveform changes are Ca++-dependent by correlating ciliary beat angles with changes in free Ca++. However, it is not know what role(s) individual OAD and IAD pathways play in these responses. Our objective is to adapt these procedures to *Tetrahymena* so that we can analyze the effects of a number of IAD and OAD mutants on Ca++-dependent waveform changes. Our results show that the beat angles of wild type *Tetrahymena* "models" also change reliably with free Ca++. Furthermore, an under-responsive IAD gene-disruption mutant called KO6 (which cannot show ciliary reversals) shows no such changes in beat angle within the physiological range of Ca++ concentrations tested while over-responsive mutants do. Our conclusion is that this procedure, along with the many IAD gene disruption mutants currently available and under construction, can be used to help understand the functions of the many different IADs in the axoneme. Supported by NSF grant MCB-0445362 to T.M.H. and NIH grant GM-59855 to D.G.P.

2417

**Young's Modulus of Primary Cilia Measured with Optical Tweezers**

Y. Harada,[1] T. Ota,[2] P. Dai,[1] H. Tanaka,[1] M. Oyamada,[1] S. Kawata,[2] T. Takamatsu[1]; [1]Department of Pathology and Cell Regulation, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Department of Applied Physics, Graduate School of Engineering, Osaka University, Osaka, Japan

Most mammalian kidney epithelial cells express solitary nonmotile primary (so-called 9+0) cilia that project into the renal tubular lumen, where they are exposed to urinary flow. The primary cilium of renal epithelia has been hypothesized to be mechanically sensitive to urinary flow. However, the mechanical properties of the primary cilium of an individual cell are not well understood. In this study, we successfully manipulated micron-sized primary cilia of MDCK cells, a widely used cell line derived from the collecting duct of canine kidney, by using optical tweezers. Optical tweezers employ the radiation pressure from a focused laser beam to trap and manipulate small objects, and forces on the objects in the trap can be measured. The primary cilia of MDCK cells, cultured on thin flexible Formvar membranes, could be clearly visualized from the side by video microscopy. Using optical tweezers, we demonstrated that we could apply forces in the piconewton range to a polystyrene bead attached to the tip of the primary cilium; thereby the primary cilium could be bent, stretched, and even snapped. We plan to report the modulus of elasticity and Young's modulus of each primary cilium, analyzed by measuring the displacement of the laser-trapped bead.

2418

**The Counterbend Response of Mouse Sperm: Evidence That the Nexin Links Have an Elastic Limit**

K. A. Lesich, C. B. Lindemann; Biological Sciences, Oakland University, Rochester, MI

Mammalian sperm demembranated with Triton X-100 and inhibited with 50 µM $NaVO_3$ in the presence of 0.1 mM ATP are immotile and flaccid. When the proximal portion of the flagellum of these sperm is manipulated into a bend with a glass microprobe, the distal flagellum develops a bend in the direction opposite the imposed bend. This is the counterbend response. An analysis of this phenomenon in rat sperm (Lindemann et al., 2005, Biophys. J. Vol. 89:1150-1164) concluded that the counterbend can best be explained by the presence of inter-doublet elastic elements in the axoneme, most likely the nexin links. Mouse sperm also exhibit the counterbend response. In addition, mouse sperm are unique in their exaggerated response to calcium. Reactivated mouse sperm respond to the addition of 1 mM $CaCl_2$ by going into a coiled shape best described as a "curlicue". The curlicue shape can generate a shear angle at the flagellar tip as large as 16 radians. This amount of shear will produce an interdoublet sliding of 800 nm between doublets pairs 2-3, 3-4, 7, 8 and 8-9 in the distal flagellum. We hypothesized that stretching the interdoublet linkages this much would likely exceed the elastic limit and result in breakage. When reactivated mouse sperm were induced into the curlicue configuration with $Ca^{2+}$, we subsequently found that they would not show a normal counterbend response after $NaVO_3$ treatment. This confirmed the hypothesis that the interdoublet elastic linkages have an elastic limit and will break if this limit is exceeded. Supported by NSF grant MCB-0110024.

2419

**A Flagellar Oscillation Driven by Mechanical Feedback**

D. M. Woolley; Physiology, University of Bristol, Bristol, United Kingdom

Eukaryotic flagella typically generate bends that alternate in direction. The mechanism of this oscillation is unknown. Here, the behaviour of quail spermatozoa in viscous media has been analysed. The flagellum of this cell has the advantages of great length, simple '9+2' structure and positional markers. When the sperm head attaches to glass, the normal 3-D flagellar motion is converted to torsion-free planar bending. Because of the high viscous resistance, fully-formed bends do not propagate in relation to an external reference-frame; instead, the flagellum is driven forward through essentially static bends. The flagellar velocity is closely correlated with the oscillation frequency. The bend-initiation-site is gradually displaced proximally, showing that all regions can act as oscillators. A meander-wave develops. Eventually the oscillation pauses. The timing of the pause is contingent on the position of the last-antecedent bend. A new bend cannot form if the direction of the thrust generated by the last bend is too

oblique. A new phase of oscillation and meander formation can be initiated by the arrival of one or more apparently spontaneously-formed, sigmoid bends from the flagellar base. Such bends decelerate as they propagate; only when they reach full development does the new oscillation begin. Sometimes, when the meander bends escape compression by slipping alongside one another (figure-of-8 mode), the oscillation continues without long pauses. These facts indicate that the last-formed bend times and regulates the oscillation by applying a bending force to the region of flagellum proximal to it. This mode of oscillation and bend propagation, dependent on a longitudinal force directed along the axonemal axis, has a close parallel in the published accounts of the behaviour of axoplasmic microtubules translocating over a glass substrate.

## Cell Motility IV (2420-2448)

2420

### DH-domain Mutation in Bcr-Abl Reveals a Synergistic Mechanism between GEFs

J. Chasseriau,[1] A. El Ali,[1] J. Rivet,[2] B. Constantin,[1] A. Kitzis,[1,2] N. Bourmeyster[1,2]; [1]Institut de Physiologie et Biologie Cellulaires UMR 6187, CNRS, Poitiers, France, [2]Laboratoire de Génétique Cellulaire et Moléculaire, CHU de Poitiers, Poitiers, France

Bcr-Abl is a chimeric oncogene found in several hematological diseases. The most frequent form is $p210^{bcr-abl}$ responsible for chronic myelogenous leukemia. Less frequent, $p190^{bcr-abl}$ is associated with acute lymphoid leukemia. The only structural difference between $p190^{bcr-abl}$ and $p210^{bcr-abl}$ resides in the absence in p190 of a DH/PH domain able to activate Rho family GTPases. In a previous work, we showed that Rho GTPases are differentially activated by both forms of Bcr-Abl. $p210^{bcr-abl}$ activates RhoA, Rac1 and Cdc42, while $p190^{bcr-abl}$, although devoid of a DH domain activates only Rac1 and Cdc42, but not RhoA. We showed that $p95^{vav}$ is present in an activated state in complex with Bcr-Abl. Our results also show that Bcr is a GEF for RhoA only, and Vav a GEF for Rac1 only. We investigated the regulation of GEF activities in the Bcr-Abl complex. Different mutants of Vav (dominant positive-DP and negative-DN mutant for GEF activity) were constructed and stably transfected in Bcr-Abl expressing Ba/F3 cells. A DH domain mutant of $p210^{bcr-abl}$ was constructed and stably expressed in Ba/F3 cells. Mobility in 3D matrigel was observed by videomicroscopy along within Boyden chamber, and GEF activity measurements were conducted. VavDN mutant inhibits pseudopodia production and Rac1 activation, suggesting that Vav is the unique vector of Bcr-Abl-induced Rac1 activation. Interestingly, in $p210^{bcr-abl}$/VavDN expressing cells, RhoA activity is lowered and amoeboid movements slow down. VavDP mutant enhances pseudopodia production and raises the level of activated Rac1. In parallel, amoeboid movements are accelerated and the level of activated RhoA is enhanced. Mutation of DH domain of $p210^{bcr-abl}$ totally inhibited the amoeboid movements, and abolished RhoA activation. A decrease in Rac1 activation was observed in these cells. A synergistic mechanism between GEF activities linked to Bcr-Abl is revealed by these experiments.

2421

### A Small GTPase Rap1 is Activated by IGF-I in Breast Cancer

W. S. Y. Lee, M. A. Guvakova; University of Pennsylvania, Philadelphia, PA

In this pilot study, we investigated a mechanism by which Rap1, a Ras family GTPase and an essential regulator for normal and some pathological cellular functions is controlled in the mammary cells. We report high levels of the Rap1 protein expression in human nontumorigenic MCF-10A cells and a number of human breast cancer cell lines, including MCF-7, T47D, BT-20, MDA-MB-231. Insulin-like growth factor I (IGF-I), a peptide known to play a role in cancer cell migration, induces an acute, but transitory increase in GTP-loading of Rap1, as we show by a Rap1 pull-down assay. The tyrosine kinase activity of the cell surface IGF-I receptor (IGF-IR) is required for Rap1 activation by IGF-I, because there was no increase in GTP-loading of Rap1 in IGF-I-treated cells expressing a dominant negative IGF-IR (IGF-IR/YF3). We then used a novel *in vivo* tool, which was generated by fusing the enhanced green fluorescent protein (EGFP) to the Rap1 binding domain of RalGDS (RBD-RalGDS), and which is recruited specifically to sites of the increased Rap1 activity in live cells. By confocal laser scanning microscopy, we monitored dynamics of EGFP-RBD-RalGDS and found accumulation of the activated Rap1 in ruffling lamellipodia of MCF-7 cells within 5 minutes of IGF-I exposure. This suggests a potential role for Rap1 in IGF-I-stimulated cell migration. Although the specific effector mechanism used by the IGF-IR to control Rap1 remains unknown, our results strongly suggest the involvement of a putative IGF-IR kinase-dependent Rap1 guanine exchange factor (GEF) acting at the plasma membrane.

2422

### Nek3 Kinase Regulation of Vav2 and Paxillin During Prolactin-mediated Breast Cancer Motility

S. L. MIller,[1] C. V. Clevenger[2]; [1]Cell and Molecular Biology, Unniversity Of Pennsylvania Medical School, Philadelphia, PA, [2]Department of Pathology, Northwestern University, Chicago, IL

Our lab has demonstrated a role for the serine/threonine kinase activity of Nek3 in the regulation of the Vav2 guanine nucleotide exchange factor during prolactin (PRL) receptor signaling. In breast cancer cell lines, PRL stimulates the generation of membrane ruffles, stress fibers and the formation of focal adhesions Given these actions of PRL and Nek3, we hypothesized that Nek3 may contribute to PRL-mediated pathogenesis and motility of human breast cancer. Initial studies using breast tissue microarrays have demonstrated a significant up-regulation of Nek3 expression in malignant versus normal specimens. Overexpression of Nek3 in CHO transfectants potentiated cytoskeletal re-organization in response to PRL. In contrast, down-regulation of Nek3 expression by small-interfering-RNA (siRNA) attenuated PRL-mediated cytoskeletal re-organization, activation of GTPase Rac1, and cell migration of the well-differentiated T47D, but not of the poorly differentiated MDA-MB-231 cells. In addition, PRL stimulation induced an interaction between Nek3 and paxillin and led to a significant increase in paxillin serine phosphorylation. Correspondingly, Nek3 siRNA transfected cells showed a marked reduction in paxillin phosphorylation. Taken together, these data suggest that Nek3 contributes to PRL-mediated breast cancer motility through mechanisms involving Rac1 activation and paxillin serine phosphorylation.

2423

### Role of the Rac Small Gtpase in Rheumatoid Arthritis

A. Chan,[1] M. Akhtar,[2] Y. Zheng,[3] P. Gulko,[4] M. Symons[1]; [1]Oncology and Cell Biology, Institute for Medical Research at North Shore-LIJ, Manhasset, NY, [2]Department of Pharmaceutical Sciences, St. John's University, Queens, NY, [3]Division of Experimental Hematology, Children's Hospital Research Foundation, Cincinati, OH, [4]Center for Genomics and Human Genetics, Institute for Medical Research at North Shore-LIJ,

Manhasset, NY
Rheumatoid arthritis (RA) is characterized by synovial hyperplasia and joint destruction. Activated fibroblast-like synoviocytes (FLS) play a critical role in RA pathogenesis. The signaling mechanisms that are responsible for the activated phenotype of RA-FLS to a large extent remain to be elucidated. Our previous work has demonstrated that the Rac small GTPase plays an important role in fibroblast invasion, proliferation and survival. We therefore hypothesized that Rac contributes to RA by stimulating the invasive and proliferative properties of FLS. We inhibited Rac function in primary FLS cultures obtained from RA patients using two independent methods, transient transfection of Rac1-directed small interfering RNA or treatment with a Rac-specific small molecule inhibitor (PNAS 101: 7618-23). Both methods yielded similar results. We showed that inhibiting Rac causes a significant inhibition in RA-FLS proliferation, without affecting cell survival. Targeting Rac also inhibited RA-FLS invasion through reconstituted extracellular matrix. Together, these results indicate that Rac contributes to the aggressive behavior of fibroblast-like synoviocytes in rheumatoid arthritis. This work was supported by an Innovative Research Grant from the Arthritis Foundation, Long Island chapter.

2424

**S100A4/Mts1 Mediated PhosphoERK and BMP2 Induced Chloride Intracellular Channel 4 (CLIC4) Produce Co-dependent Migration of Human Pulmonary Artery Smooth Muscle Cells (hPASMCs)**

E. Spiekerkoetter,[1] L. Wang,[1] N. Ambartsumian,[2] A. Schmidt,[3] M. Rabinovitch[1]; [1]Stanford University Medical School, Stanford, CA, [2]Danish Cancer Society, Copenhagen, Denmark, [3]Department of Physiology, Medicine and Surgery, Columbia University, New York, NY

Mutations in bone morphogenetic protein receptor (BMPR)-II are linked to pulmonary hypertension (PH). Our previous studies showed that migration of hPASMCs, a key feature in PH, was induced by BMP2 (a ligand for BMPR-II) and S100A4/Mts1 (the ligand for the receptor for advanced glycation end products (RAGE)) in an interdependent manner. That is, migration of hPASMCs induced by Mts1 or BMP2 was inhibited by either anti-RAGE or BMPR-II siRNA. Further characterization of this interdependency is important in understanding the link between the mutation and the disease. We therefore investigated the signaling pathways and gene expression that require input from RAGE and BMPR-II after stimulation with Mts1 or BMP2. We treated hPASMCs with recombinant (r)Mts1 (500 ng/ml) or BMP2 (10 ng/ml) ± preincubation with a RAGE blocking antibody ± BMPR-II siRNA. Migration was quantified in a modified Boyden chamber. Activation of signaling pathways was evaluated by western immunoblot and changes in gene expression by cDNA microarray analysis and quantitative RT-PCR, followed by siRNA. Phosphorylation of ERK (phosphoERK), induced by both ligands, was blocked by anti-RAGE but not by BMPR-II siRNA ($n=3$, $p < 0.001$) suggesting phosphoERK is necessary but not sufficient to induce migration. BMPR-II siRNA (70% knock-down), but not anti-RAGE led to downregulation of CLIC4 gene expression to < 40% ($n=3$, $p < 0.001$). Knocking down CLIC4 by siRNA (80% knock-down) abrogated Mts1 and BMP2 induced migration ($p < 0.001$, $n=4$). We conclude that migration of hPASMCs with either Mts1 or BMP2 requires ERK activation via RAGE and CLIC4 expression via BMPR-II. We speculate that when there is BMPR-II loss of function, another stimulus is necessary to de-repress CLIC4 permitting smooth muscle cell migration associated with PH pathology.

2425

**Paxillin-associated ARF-GAP Activity Mediates Slit2-Robo4 Induced Chemorepulsion**

N. Nishiya,[1] C. A. Jones,[2] W. B. Kiosses,[3] J. Han,[1] D. Y. Li,[2] M. H. Ginsberg[1]; [1]Medicine, University of California San Diego, La Jolla, CA, [2]Program in Human Molecular Biology and Genetics, University of Utah, Salt Lake City, UT, [3]The Scripps Research Institute, La Jolla, CA

Attractive and repulsive guidance cues orchestrate pathfinding events in the developing nervous, vascular, and other systems. Directional cell migration requires inhibition of Rac activation at the trailing edge to maintain cell polarity. In the present study, we establish a general mechanism by which membrane receptors attenuate Rac activation, thereby resulting in repulsion. Previously, we found (Nature Cell Biol. vol 7, pg 343, 2005) that the alpha4 integrin subunit recruits paxillin-binding ARF-GAPs to the rear of a migrating cell, leading to local inhibition of Rac activation and suppression of membrane protrusive activity. We now report that this pathway mediates chemorepulsion involved in vascular patterning. Robo family chemorepulsive receptors are transmembrane receptors that regulate pathfinding events. Among the family, Robo4 is specifically expressed in endothelial cells and controls the trajectory of inter somitic angiogenesis . Robo4 inhibits endothelial cell migration through binding of a chemorepulsive ligand, Slit2. We report that paxillin and its paralogue, HIC-5, bind to the cytoplasmic domain of Robo4. Paxillin binding to Robo4 leads to recruitment of an ARF-GAP and consequent inhibition of Rac activation and cell protrusion in response to Slit2. These data establish the generality of this novel pathway for regulating Rac activity and show that the signaling pathway can mediate developmental chemorepulsion in addition to contributing to the polarization of migrating cells.

2426

**A New Positive Role for Raf Kinase Inhibitor Protein in Epithelial Cell Migration**

S. Zhu, K. T. Mc Henry, G. Fenteany; Chemistry, University of Illinois, Chicago, IL

Raf kinase inhibitor protein (RKIP) is a modulator of cell signaling that functions as an endogenous inhibitor of multiple kinases. Here we demonstrate a new positive role for RKIP in the regulation of cell locomotion. We discovered that RKIP is the relevant cellular target of locostatin, a novel cell migration inhibitor. Locostatin abrogates RKIP's ability to bind and inhibit Raf-1 kinase, making it the first known inhibitor of RKIP, one that acts by disrupting a protein-protein interaction, an uncommon mode of action for a small molecule. Small interfering RNA-mediated silencing of RKIP expression also reduces cell migration rate. Overexpression of RKIP converts epithelial cells to a highly migratory fibroblast-like phenotype, with dramatic reduction in the sensitivity of cells to locostatin. RKIP is therefore the compound's valid target and a key regulator of cell motility.

2427

**Targeting and Activation of Rac1 are Mediated by the Exchange Factor β-Pix**

P. L. Hordijk, J. ten Klooster; Experimental Immunohematology, Sanquin Research, Amsterdam, The Netherlands

Rho GTPases are critical regulators of cytoskeletal dynamics and control complex functions such as cell adhesion, spreading, migration and cell division. It is generally accepted that localized GTPase activation is required for proper initiation of downstream signalling events, although the

molecular mechanisms that control intracellular targeting of Rho GTPases are unknown. Rho GTPases carry a C-terminal lipid anchor for membrane association, but likely also require protein domain(s) for specific, subcellular targeting. Here we show that the Rho GTPase Rac1, via a proline stretch in its C-terminus, binds to the SH3 domain of the Cdc42/Rac activator β-Pix. This interaction with endogenous β-Pix is necessary and sufficient for Rac1 recruitment to the plasma membrane and to focal adhesions and for Rac1 activation by β-Pix. Moreover, we show that Rac1 and Pak1, are competitive interactors for β-PIX. Finally, activated CDC42 was found to promote the β-PIX-mediated recruitment of Rac1 to the membrane, possibly by activating PAK1, which leads to its dissociation from β-PIX.. These data provide a model for the intracellular targeting and localized activation of Rac1 through a Cdc42-Pak-β-Pix axis.

2428

**Components of Sema3A Receptor Complex Implicated in the Migration of Glioma Cells**

C. Pinaudeau (Nasarre), M. Perraut, D. Aunis, D. Bagnard; Centre de Neurochimie, INSERM U575, Strasbourg, France

Recent studies showed that semaphorins may play critical roles in cancer cells migration, and may act in the migration of glial cells. Here, using co-culture assays, we investigated the biological effects of a secreted class 3 semaphorin (Sema3A) on the migration of C6 glioma cells, a model of the major human brain tumour cells. Our results demonstrated that Sema3A had no effect on the migration on C6 cells after 24 hours co-culture. However, a significant chemorepulsive effect occurred after 48 hours, and the effect increased with time, indicating that the expression of neuropilin-1 (NRP1), the ligand-binding subunit of semaphorin receptor complex, might be long-term up regulated by the presence of Sema3A. Indeed, the expression of NRP1 was weak in C6 cells; however, by the presence of Sema3A, the NRP1 expression increased. The expression of NRP2 (a potential binding subunit of Sema3A), and the expression of Plexine-A1 (Plex-A1; the functional receptor of Sema3A), were not modified by the presence of Sema3A. Gain of function experiments in which NRP1 was over-expressed in C6 cells also showed a significant chemorepulsive effect of Sema3A. These results suggested that the repulsive effect of Sema3A was dependant of NRP1. Then, using blocking antibodies against NRP1 or Plexine-A1, we showed that the chemorepulsive effect of Sema3A wasn't only lost, but strikingly, was switched into a chemoattractive one. With a blocking antibody against NRP2, NRP1+NRP2, NRP2+Plex-A1 or NRP1+NRP2+Plex-A1, the repulsive effect was abolished. These results confirmed that the repulsion mediated by Sema3A in the C6 cell migration required a receptor complex composed of at least 3 subunits NRP1/NRP2/Plex-A1, in which NRP2 might be a modulator. Indeed, in C6 cells overexpressing NRP2, Sema3A had a chemoattractive effect.

2429

**Mechanisms of Regulation of GIT1 Activation by PIX Required for Cell Migration**

L. Za, S. Paris, A. Totaro, I. de Curtis; San Raffaele Scientific Institute, Milano, Italy

Cell migration requires the reorganization of the cytoskeleton and membrane trafficking for effective protrusion. The mechanisms coordinating these processes are poorly understood. GIT1/p95-APP1 is an ubiquitous ArfGAP that forms stable complexes with the Rac activator/effector complex PIX/PAK, and with paxillin. Studies in neuronal and non neuronal cells indicate an important role of this complex in the regulation of endocytosis and focal adhesion assembly in migrating cells. We have shown by time lapse analysis that motogenic factors induce the rapid accumulation of the GIT mutants at perinuclear endocytic structures, causing inhibition of lamellipodia formation and cell retraction. BetaPIX is required for the association of the GIT1 complex to transferrin receptor-positive endocytic structures. Both dimerization and a functional SH3 domain are necessary for this function of betaPIX. The interaction of betaPIX with GIT1 correlates with the release of an intramolecular interaction between the aminoterminal and carboxyterminal portions of GIT1, and with increased binding to paxillin, a protein implicated in the regulation of integrin-mediated adhesion. Immunofluorescence and cell fractionation were use to establish the tight association of about 30% of the endogenous complex to membranes. Moreover, studies with permeabilized cells show a correlation between the association of endogenous betaPIX to GIT1 and the association of the endogenous complex to membranes. Our data indicate that _etaPIX is an important regulator of the subcellular distribution and regulation of GIT1. BetaPIX-mediated relocalization of the complex allows the redistribution of factors important for the regulation of adhesion and actin dynamics during motility. Supported by Telethon-Italy, grant GGP02190.

2430

**HGF-mediated Endothelial Cell Migration Requires c-Met Ligation, $S1P_1$ and EphA2 Receptor Transactivation, Dynamin2, Cortactin, PI3 kinase and Rac1**

P. A. Singleton, S. M. Dudek, J. G. N. Garcia; Medicine, University of Chicago, Chicago, IL

Therapeutic angiogenesis is an important goal in the treatment of atherosclerosis and ischemic heart failure. As hepatocyte growth factor (HGF) ligation of its receptor tyrosine kinase, c-Met, invokes a potent angiogenic response, we examined the mechanism(s) by which HGF promotes human endothelial cell (EC) migration, a key component of the angiogenic response. We observed that HGF (50 ng/ml, 5 min.) induced c-Met tyrosine phosphorylation, threonine phosphorylation of the sphingosine 1-phosphate receptor ($S1P_1$)and tyrosine phosphorylation of EphA2 receptor in caveolin-enriched EC microdomains (CEM, or lipid rafts). Further, HGF induced recruitment to CEMs of the vesicular regulatory molecule, dynamin II, the actin-regulatory molecule, cortactin, and the regulatory PI3 kinase subunit, p85α in concert with Rac1 activation. Reductions in c-Met or $S1P_1$ expression (siRNAs) blocked HGF-induced EphA2 tyrosine phosphorylation, activation of Rac1, and CEM recruitment,of dynamin II, cortactin, and p85α PI3 kinase. Reductions in EphA2 expression did not block HGF-induced c-Met or $S1P_1$ phosphorylation but attenuated p85α PI3 kinase CEM recruitment and Rac1 activation. Reductions in dynamin II, but not cortactin expression further attenuated HGF-induced Rac1 activation. Finally, attenuation of either lipid raft formation (via cholesterol depletion), actin cytoskeletal rearrangement (via cytochalasin D) or c-Met, $S1P_1$, EphA2, dynamin II, cortactin, p85α PI3 kinase or Rac1 expression all served to block HGF-induced EC migration. These results indicate that c-Met transactivation of $S1P_1$ receptor and EphA2 receptor in CEM is required for HGF-induced EC migration.

2431

**Regulation of VASP Serine 157 Phosphorylation in Human Neutrophils Stimulated by Chemoattractants**

R. E. Eckert, Y. Sharief, S. L. Jones; Clinical Sciences, North Carolina State University, Raleigh, NC

Vasodilator-stimulated phosphoprotein (VASP) is a cAMP-dependent protein kinase (PKA) substrate that links cellular signaling to cytoskeletal organization and cellular movement. VASP is phosphorylated by PKA on serine 157, which is required for VASP function in platelet adhesion and fibroblast motility. Our hypothesis is that PKA regulates neutrophil migration through VASP Ser 157 phosphorylation. The objective of this study was to characterize VASP Ser157 phosphorylation in chemoattractant-stimulated neutrophils. Formylated Met-Leu-Phe (fMLF), IL8, leukotriene $B_4$ ($LTB_4$), or platelet-activating factor (PAF) stimulation resulted in an initial increase in VASP Ser 157 phosphorylation that was maximal by 30s, and was followed by a return to baseline Ser 157 phosphorylation by 10 min. In contrast, stimulation with the pro-inflammatory cytokine TNF alpha did not affect Ser 157 phosphorylation. FMLF-induced Ser 157 phosphorylation was abolished by pretreatment with the PKA-specific inhibitors H89 or KT5720, but was unaffected by the PKC inhibitor staurosporine or the PKG inhibitor Rp-8-pCPT-cGMP. Moreover, prolonging PKA activity by elevating cAMP with prostaglandin $E_2$ or the phosphodiesterase inhibitor IBMX did not increase initial VASP Ser 157 phosphorylation, but did reduce VASP dephosphorylation at later time points. Inhibition of adhesion with either EDTA or the anti-beta 2 integrin antibody IB4 did not alter fMLF-induced VASP phosphorylation or dephosphorylation. These data show that chemoattractant stimulation rapidly increases PKA-dependent VASP Ser 157 phosphorylation, followed by rapid dephosphorylation. Adhesion does not appear to be an important regulator of the state of VASP Ser 157 phosphorylation in chemoattractant-stimulated neutrophils.

2432

### Regulation of Cell Migration by the Cofilin Phosphatase, Chronophin, in EGF-stimulated MTLn3 cells

C. DerMardirossian,[1] V. Delorme,[1] T. Huang,[1] G. M. Danuser,[2] C. M. Waterman-Storer[2]; [1]Immunology, The Scripps Research Institute, La Jolla, CA, [2]Cell Biology, The Scripps Research Institute, La Jolla, CA

Cell motility plays a crucial role in many basic biological processes, including development and wiring of the nervous system, wound healing, inflammation, and cancer metastasis. Motility of cells is dependent on a coordinated regulation of actin cytoskeletal dynamics, i.e. cycles of actin polymerization and depolymerization. As a crucial mediator of actin dynamics, cofilin is regulated by cycling between an active, unphosphorylated form that depolymerizes and/or severs actin, and an inactive, phosphorylated form. The inactivation of cofilin is mediated through its phosphorylation by LIM kinase and its activation results from its dephosphorylation through two unrelated phosphatases: slingshot (SSH) and a recently characterized phosphatase, chronophin (CIN). We examined the effects of this novel cofilin phosphatase, CIN, in actin cytoskeletal dynamics in the context of stimulated cell motility. We have found that in starved carcinoma cells (MTLn3), overexpressed CIN is mostly diffusely distributed in the cytosol. In response to epidermal growth factor (EGF) stimulation, a marked transient localization of overexpressed CIN wt at the leading edge of the lamellipodium occurs at 3 min. In contrast, overexpressed CIN dominant negative (D25N) remained distributed throughout the cytoplasm. Interestingly, EGF-stimulated MTLn3 does not induce SSH translocation to the leading edge. We next determined whether CIN regulates cell motility in response to growth factor. Analysis of cell speed, using phase-contrast microscopy revealed that MTLn3 injected with CIN wt migrated nearly twice as fast as cells controls. Consistent with these results, we observed marked effects of WT and DN CIN on actin dynamics at the leading edge, as determined by quantitative Fluorescence Speckle Microscopy. We are currently analyzing the mechanisms regulating CIN translocation and function.

2433

### Rac1 and Rac2 Regulate Cell Morphology But Are Not Essential for Migration

A. P. Wheeler,[1] S. D. Smith,[2] C. M. Wells,[3] V. Tybulewicz,[4] A. J. Ridley[5]; [1]Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Ludwig Institute for Cancer Research, London, United Kingdom, [3]The Ludwig Institute for Cancer Research, London, United Kingdom, [4]The National Insitute for Medical Research, London, United Kingdom, [5]Cell and Molecular Biology, The Ludwig Institute for Cancer Research, London, United Kingdom

Rac proteins are Rho family GTPases involved in transducing signals from cell surface receptors to the cytoskeleton, cell-cell and cell-substratum adhesions and cell cycle . Mammals have three closely related Rac isoforms, Rac1, Rac2 and Rac3. Rac1-null mice die early in embryogenesis, whereas Rac2 is not essential for development or fertility. Rac2 is predominantly expressed in haematopoietic cells, and Rac2-null mice show defects in the function of several different haematopoeitic cell types. Using haematopoietic cell-specific knockout of Rac1, Rac1 and Rac2 have been shown to have differential roles in differentiation, motility, adhesion, phagocytosis and recruitment . We have previously shown that loss of Rac1 in macrophages affects cell shape but not migration speed. Rac3 is not expressed in macrophages, but it is possible that Rac2 compensates for loss of Rac1. Here we report that Rac2 deletion does not affect macrophage migration speed although it does induce loss of podosomes, integrin-based adhesion sites in myeloid cells, and affects cell adhesion. Deletion of Rac1 and Rac2 alters cell morphology but surprisingly does not prevent the migration of macrophages, although it alters their mode of migration.

2434

### Arf6 Acts as a Negative Regulator of Rac1 Activity in Membrane Ruffling Formation

M. Cotton,[1] T. Houndolo,[1] J. A. Pitcher,[2] A. Claing[1]; [1]Pharmacologie, Université de Montréal, Montréal, PQ, Canada, [2]MRC Laboratory for Molecular and Cellular Biology and Department of Pharmacology, University College of London, London, United Kingdom

The ADP-ribosylation factor 6 (ARF6) and Rac1 are small GTP-binding proteins that regulate several signaling events ranging from vesicle trafficking, to modification of membrane lipids and reorganization of the actin cytoskeleton. However, the molecular mechanisms by which ARF6 and Rac1 act in concert to control these different cellular process remain unclear. Here, we show that in HEK 293 cells, ARF6 and Rac1 can be found in complex upon stimulation of the angiotensin receptor. In vitro experiments indicate that these two small G proteins can directly interact together, and that ARF6 preferentially interacts with the GDP-bound form of Rac1. Depletion of ARF6 by RNA interference leads to a marked activation of Rac1 in cells independently of receptor stimulation. Conversely, overexpression of the ARF guanine-nucleotide exchange factor ARNO significantly reduces the angiotensin-stimulated activation of Rac1 while a dominant negative form of ARNO has no effect. Unregulated Rac1 activation induced by the depletion of ARF6 has important physiological consequences. First, angiotensin-stimulation of Rac1 leads to membrane ruffling. Interestingly, depletion of ARF6 also leads to similar membrane ruffling together with the formation of actin-rich protrusions, which can be prevented by expression of a dominant negative form of Rac1. Reorganization of the actin cytoskeleton induced by suppression of ARF6 expression also leads to cell migration. Taken together, these results demonstrate that ARF6 is a key regulator of Rac1 activity *in vivo.*

2435

**Synergistic Effect of Activated Cdc42 and Inactivated Rho via PI 3-kinase Is Required for Injury-induced Cell Migration in Corneal Endothelial Cells**

J. Lee,[1] E. P. Kay[1,2]; [1]Doheny Eye Institute, Los Angeles, CA, [2]Ophthalmology, USC Keck School of Medicine, Los Angeles, CA

Acquisition of elongated cells with prominent pseudopodia is observed in CECs simultaneously treated with FGF-2 and RhoA inhibitors. In the present study, we investigated whether these pseudopodia-containing elongated CECs were migratory phenotypes and whether Cdc42 activation and RhoA inactivation were involved in cell migration via phosphatidylinositol (PI) 3-kinase. Scratch-induced directional migration assay was employed in a 90% confluent CECs; wound healing and migratory rates were measured 24 h following wounding. FGF-2 stimulation alone resulted in a 43% recovery of the wound area, but recovery was completely blocked by the addition of LY294002. CECs treated with both FGF-2 and ROCK inhibitor achieved an 84% recovery of the wound area with a fast migratory speed (0.72 μm/min), and the synergistic effects of FGF-2 and ROCK inhibitor were completely blocked by LY294002. Under these conditions, significant PI 3-kinase and Cdc42 activation was also observed in migratory cells. We further confirmed the involvement of activated Cdc42 and inactivated Rho kinase in endothelial migration by transfecting CECs with constitutively active (ca) or dominant negative (dn) vectors (Rho, Rac, or Cdc42). CECs expressing caCdc42 demonstrated a very high migratory rate (0.52 μm/min), while those expressing caRho showed a complete inhibition of endothelial migration. When cells expressing caCdc42 were treated with FGF-2, migration reached the maximum rate (0.69 μm/min), similar to that observed in cells treated with FGF-2 and Y27632. These findings suggest that endothelial migration is induced by activated Cdc42 and inactivated Rho kinase via PI 3-kinase pathway in response to FGF-2 and that Cdc42 activation is crucial to CECs acquiring characteristic endothelial migration phenotypes.

2436

**A DDR1/Shp2 Signaling Complex Inhibits α2β1 Integrin-mediated Stat1/3 Activation and Cell Migration**

C. Wang,[1] H. Su,[1] Y. Hsu,[2] M. Shen,[3] M. Tang[2]; [1]Physiology, Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan Republic of China, [2]Physiology, National Cheng Kung University, Tainan, Taiwan Republic of China, [3]Pharmacology, National Cheng Kung University, Tainan, Taiwan Republic of China

Cell migration is one important factor that regulates branching tubule morphogenesis in collagen gel. Here we showed that discoidin domain receptor 1 (DDR1) inhibited collagen-induced tyrosine phosphorylation of Stat1 and Stat3 and cell migration triggered by α2β1 integrin. Overexpression of DDR1 increased the interaction of DDR1 with SHP-2 and upregulated the tyrosine phosphatase activity of SHP-2. We demonstrated that the SH2-SH2 and PTP domains of SHP-2 were responsible for interaction with DDR1, and that both tyrosine phosphorylation sites 703 and 796 of DDR1 were essential for it to bind with SHP-2. Mutation of tyrosine 703 or 796 of DDR1 abolished the ability of DDR1 to inhibit the tyrosine phosphorylation of Stat1 and Stat3, and restored collagen-induced cell migration and hepatocyte growth factor (HGF)-induced branching tubulogenesis in collagen gel. Taken together, these results demonstrate that SHP-2 is required for the DDR1-induced suppression of Stat1/3 tyrosine phosphorylation, cell migration, and branching tubulogenesis.

2437

**Investigation of Centaurin β4 Arf-gap Isoforms in Human Melanoma Cell Lines**

R. K. Martin, T. Thody, T. R. Jackson; Dermatological Sciences, University of Newcastle, Newcastle-upon-Tyne, United Kingdom

PCR, sequencing and western blotting were used to look for evidence of alternate splicing over the C terminal proline rich regions and SH3 domain of centaurin β4. These domains are used for protein-protein interactions involving oncogenes and focal adhesion proteins. Western blotting using two different antiserum show multiple bands in melanoma cell line lysates. To determine whether these arise by alternate splicing we have performed PCR across the C terminal region known to show splicing in mouse. The total PCR mix containing bands of varying sizes were cloned and subjected to sequencing for each size band for each cell line in duplicate. Two splice forms present in all four melanoma cell lines screened are orthologous to those found in mouse. Three further novel splice variants were found in two melanoma lines. Our investigation of Centaurins in human melanoma cell lines has found several isoforms of Centaurin β4. Centaurins are ADP Ribosylation Factor (Arf) GTPase Activating Proteins (GAPs) that, together with Arf Guanine nucleotide Exchange Factors (Arf-GEFs), regulate cyclic activation of Arfs. Arf signalling is required for certain actin cytoskeletal responses and intracellular trafficking. Centaurin β4/ASAP1/DDEF1/PAG3/AMAP1, shows Arf-GAP activity towards Arf1 and Arf5 . It is the product of the DDEF1 gene locus which has recently been shown to be amplified and upregulated in different stages of uveal melanoma , breast and colon cancer . Ddef1 has also been reported to drive adipogenic differentiation . These novel human melanoma splice forms variously lack proline rich regions implicated in interactions with Src , Crk-L and cortactin , or an SH3 domain implicated with paxillin and FAK , POB1 and Pyk2 . These may therefore differ in their regulation of adhesion and therefore metastatic properties and/or cell growth.

2438

**Identification of Par3 as a Novel Substrate of Rho-kinase**

M. Nakayama,[1] T. Nishimura,[1] M. Sugimoto,[2] T. M. Goto,[1] S. Ohno,[3] M. Amano,[1] K. Kaibuchi[1]; [1]Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Vascular Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan, [3]Department of Molecular Biology, Yokohama City University School of Medicine, Yokohama, Japan

The Rho/Rho-kinase pathway plays important roles in various cell events including cell contraction, cell migration, and polarity establishment. Various substrates of Rho-kinase were already identified so far, such as myosin-binding subunit (MBS) of myosin phosphatase, myosin light chain (MLC), Adducin, Ezrin/Radixin/Moesin (ERM), Lim-kinase, Calponin, and Map2/Tau, which regulate the actin cytoskeleton and microtubules. However, the substrate which regulates cell polarity establishment has not yet been identified. To understand roles of the Rho/Rho-kinase pathway in establishment of cell polarity precisely, we searched for the other Rho-kinase substrates by in vitro kinase assay and identified Par3 as a novel substrate of Rho-kinase. Par3 forms the large protein complex with Par6, atypical protein kinase (aPKC), Cdc42, and so on. This complex functions in various cell-polarization events, including axon specification, asymmetric cell division, cell migration and cell morphology. We determined phosphorylation sites of Par3 by Rho-kinase and also found that interactions among Par complex components, Par3, Par6 and aPKC, were disrupted by phosphorylation of Par3 by Rho-kinase, which decreased the affinity of Par6 and aPKC to Par3. Our results suggest that Rho-kinase plays important roles in cell polarity formation through regulating Par complex.

2439

**Cdc42 is Required for EGF-Stimulated Protrusion and Motility in Carcinoma Cells**

M. El-Sibai,[1] P. Nalbant,[2] F. Macaluso,[3] M. Cammer,[3] M. Symons,[4] J. S. Condeelis,[5] K. M. Hahn,[6] J. M. Backer[1]; [1]Molecular Pharmacology, Albert Einstein College of Medicine, The Bronx, NY, [2]Immunology, Scripps Research Institute, La Jolla, CA, [3]Analytical Imaging Facility, Albert Einstein College of Medicine, The Bronx, NY, [4]Center for Oncology and Cell Biology, Institute for Medical Research at North Shore, Manhasset, NY, [5]Anatomy and Cell Biology, Albert Einstein College of Medicine, The Bronx, NY, [6]Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC

Cdc42 plays a central role in regulating the actin cytoskeleton and maintaining cell polarity. In this study, we have used a recently developed Cdc42 biosensor (MeroCBD) in order to monitor the activation of endogenous Cdc42 in single cells. When stimulated with EGF, carcinoma cells showed a rapid increase in activated Cdc42 that is primarily localized to the leading edge of the cells. Cdc42 activation is co-temporal with the actin polymerization transients at 1 and 3 min previously defined in MTLn3 cells. To identify the upstream regulator of Cdc42, we examined the effect of the PI3K inhibitor wortmannin on Cdc42 activity. PI3K inhibition led to a substantial decrease in Cdc42 activation. Surprisingly, siRNA-mediated knock-down of Cdc42 expression caused a decrease in EGF-stimulated PIP3 production, suggesting a positive feedback mechanism coordinately regulating Cdc42 and PI3K. We next looked at the effect of Cdc42 inhibition on cell protrusion and lamellopodia formation. Cells treated with Cdc42 siRNA duplexes showed a decrease in EGF-stimulated protrusion. This decrease correlated with a decrease in Arp2/3 localization at the cell edge. The loss of Arp2/3 recruitment in Cdc42 siRNA-treated cells was consistent with images obtained by rotary shadowing scanning electron microscopy, which showed a marked defect in actin filament branching. Finally, we found that the Cdc42 siRNA-treated cells show reduced motility in time-lapse studies. Our data show that the PI3K-kinase-dependent activation of Cdc42 at the leading edge is required for both protrusion and motility in carcinoma cells.

2440

**Role of Rac1 in Heregulin-Mediated Cell Invasion**

S. J. Coniglio,[1,2] S. R. Nanda,[3] M. H. Symons[1,2]; [1]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]Institute for Medical Research at North Shore-LIJ, Manhasset, NY, [3]Department of Biology, St. Johns University, Queens, NY

The Rho-GTPase Rac1 has been shown by our laboratory to be critical for cell invasion in a panel of human tumor lines. The mechanism of Rac1 mediated invasion however is unclear. The purpose of this study is to investigate the molecular mechanism(s) responsible for invasion downstream of Rac1. Here we show Rac1 is required for the heregulin (Hrg)-stimulated lamellipodia and invasion of human breast carcinoma cell line T47D. Hrg potently activates many signaling pathways potentially involved in invasion and carcinogenesis including ERK, JNK, p38, and PI-3K/AKT. Using small interfering RNA (siRNA), we demonstrate that Rac1 is required for Hrg-mediated activation of JNK1 and JNK2, whereas activation of ERK and p38 are largely independent of Rac1. The mammalian group 1 Pak kinases (Pak 1-3) are direct effectors of Rac and Cdc42. Activation of both Pak1 and Pak2 by Hrg is mediated primarily by Rac1, with a minor contribution by Cdc42. Inhibition of either Pak1 or Pak2 using siRNA blocks Hrg-stimulated invasion. Furthermore, blockade of the JNK pathway with the pharmacological inhibitor SP600125 also inhibits invasion. In summary, we propose a model whereby Hrg stimulates cell invasion via Rac1, Pak1, Pak2 and JNK.

2441

**Live Visualization of RhoA Activity in Carcinoma Cells Reflects a Novel Role of the Rho/p160ROCK in Cell Motility**

M. El-Sibai,[1] O. Pertz,[2] M. Lorenz,[3] J. S. Condeelis,[3] K. M. Hahn,[4] J. M. Backer[1]; [1]Molecular Pharmacology, Albert Einstein College of Medicine, The Bronx, NY, [2]Immunology, Scripps Research Institute, La Jolla, CA, [3]Anatomy and Cell Biology, Albert Einstein College of Medicine, The Bronx, NY, [4]Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC

RhoA plays a central role in cell motility through the regulation of adhesion and actin/myosin contractility. Recently, RhoA has been found to localize to the leading edge of moving fibroblasts, suggesting a role of RhoA in lamellopodia formation. In this study, we use a FRET-based RhoA biosensor in order to examine the spatial and temporal activation of RhoA in EGF-stimulated carcinoma cells (MTLn3). We found that RhoA follows a biphasic activation profile with peaks at 1 and 3 min, coinciding with the dynamics of EGF-stimulated actin polymerization in MTLn3 cells. The activation of RhoA was localized to a 5 μm-wide zone at the leading edge of cells, suggesting that Rho helps to coordinate the regulation of actin nucleating proteins leading to lamellopodia formation. Inhibition of Rho by microinjection of cells with C3 exoenzyme led to a constitutive increase in cell area. In contrast, cells treated with the ROCK inhibitor Y27632 showed normal basal surface area, but showed an exaggerated extension of lamellopodia in response to EGF. Inhibition of Rho or ROCK also led to a change in Rac localization; instead of accumulating in a narrow band at the edge of control EGF-stimulated cells, Rac was localized in a broad 5 μm band in C3T- or Y27632 treated cells. Inhibition of Rho or ROCK caused a decrease in cell motility in serum that correlated with a decrease in myosin light chain phosphorylation and focal adhesion formation following EGF stimulation. Our study shows that the antagonistic effect of Rho on Rac at the leading edge is mediated through ROCK, and suggests that the RhoA/p160ROCK pathway regulates the formation of adhesive structures during EGF-stimulated protrusion.

2443

**Diacylglycerol Kinase-ζ Regulates Growth Factor-Induced Membrane Ruffling and Migration by Promoting Rac1-RhoGDI Dissociation and PAK1 Phosphorylation**

H. Abramovici,[1,2] R. Parks,[1,3] M. K. Topham,[4,5] X. P. Zhong,[6,7] G. A. Koretzky,[6,8] S. H. Gee[1,2]; [1]Center for Neuromuscular Disease, University of Ottawa, Ottawa, ON, Canada, [2]Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, [3]Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada, [4]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, [5]Internal Medicine, University of Utah, Salt Lake City, UT, [6]Signal Transduction Program, Abramson Family Cancer Research Institute, Philadelphia, PA, [7]Cancer Biology, University of Pennsylvania, Philadelphia, PA, [8]Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Growth factor-induced cell migration relies on the rapid reorganization of the actin cytoskeleton, a process regulated by the small Rho GTPases, Rac1, RhoA and Cdc42. Rac1 controls the formation of lamellipodia and membrane ruffles, specialized structures that promote cell movement. Although Rac1 associates with several signaling and scaffolding proteins, relatively little is known about how these proteins regulate its function. Here, we show that diacylglycerol kinase-ζ (DGK-ζ), an enzyme that phosphorylates diacylglycerol to yield phosphatidic acid, associates with

Rac1 and plays an important role in membrane ruffling and cell migration in mouse embryonic fibroblasts. DGK-ζ deficient (-/-) fibroblasts showed a three-fold decrease in platelet-derived growth factor (PDGF)- induced membrane ruffling. In addition, (-/-) fibroblasts displayed a 50% decrease in migration rate in both wound healing and three-dimensional migration assays. The decrease in ruffling and migration could be rescued by infecting DGK-ζ (-/-) fibroblasts with adenovirus containing wild-type DGK-ζ, suggesting DGK-ζ plays a role in regulating Rac1-dependent actin remodeling. Consistent with this interpretation, overexpression of a DGK-ζ mutant lacking the Rac1-binding C1AC1B domains decreased membrane ruffling in wild-type (+/+) fibroblasts. Compared to DGK-ζ (-/-) cells, PDGF-stimulated (+/+) fibroblasts had increased Rac1 activity and a decreased amount of Rac1 associated with the Rho guanine-nucleotide dissociation inhibitor (RhoGDI), which sequesters and inhibits Rac1. DGK-ζ (+/+) cells also had higher levels of phosphorylated PAK1, a Rac1-regulated serine/threonine kinase involved in membrane ruffling and migration. Taken together, these results suggest that DGK-ζ regulates Rac1-dependent actin remodeling by promoting the dissociation of Rac1 from RhoGDI and increasing the phosphorylation and activity of PAK1.

2444

**Serine 2 Phosphorylation Regulates Coronin 1B Activity and Affects Cell Migration**

L. Cai,[1,2] M. D. Schaller,[1,2] J. E. Bear[1,2]; [1]Cell and Developmental Biology, School of Medicine, UNC at Chapel Hill, Chapel Hill, NC, [2]Lineberger Comprehensive Cancer Center, Chapel Hill, NC

Coronin 1B, a conserved WD40 repeat-containing, actin binding protein, is ubiquitously expressed and co-localizes with the Arp2/3 complex at the leading edge of fibroblasts. Pharmacological experiments show that the interaction between Coronin 1B and the Arp2/3 complex is regulated through protein kinase C (PKC) phosphorylation. Using tryptic peptide mapping and site-direct mutagenesis, we show that Coronin 1B is phosphorylated at Serine 2 (Ser2) upon phorbol-12-myristate-13-acetate (PMA) stimulation *in vivo*. Rat2 fibroblasts expressing the Coronin 1B S2A mutant show enhanced ruffling in response to PMA and increased speed in single cell tracking assay, while the S2D mutant expression attenuates PMA-induced ruffling and slows cell speed. Expression of the S2A mutant partially protects cells from the inhibitory effects of PMA on cell speed. These data demonstrate that Coronin 1B regulates leading edge dynamics and cell motility in fibroblasts through Ser2 phosphorylation, and this phosphorylation is responsible for a measurable fraction of PMA's effects on motility. To elucidate the regulation of Coronin 1B function via Ser2 phosphorylation, we developed an assay for measuring dephosphorylation using a phosphor-specific antibody. Following termination of PMA treatment with PKC inhibitors, phosphorylated Coronin 1B undergoes a rapid, okadaic acid-insensitive dephosphorylation and returns to basal phosphorylation level within 10 min. RNAi-mediated knockdown of Coronin 1B leads to defects in both leading edge protrusion and cell motility, which can be rescued by over-expression extrinsic wild-type protein. While S2A and S2D mutants reciprocally enhance or suppress cell motility in the presence of wild-type protein, neither is capable of rescuing Coronin 1B knockdown phenotypes. These data are consistent with a model in which Coronin 1B undergoes cycles of phosphorylation and dephosphorylation *in vivo* in order to function properly.

2445

**CSF-1-stimulated Wasp Activation is PI 3-kinase and Cdc42-dependent**

M. Cammer,[1] R. House,[2] M. Lorenz,[3] J. Condeelis,[1] D. Cox[1]; [1]Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]Biochemistry, Albert Einstein College of Medicine, Bronx, NY, [3]Optical Technology Development, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

A role for WASP (Wiskott-Aldrich Syndrome Protein) in chemotaxis to various chemoattractants has been demonstrated in monocyte-derived macrophages, dendritic cells and osteoclasts, although it is unclear how WASP activity is regulated in vivo. To determine the temporal and spatial activation of WASP, we have constructed a WASP biosensor (WASPbs) that exhibits intramolecular fluorescence resonance energy transfer (FRET) in its autoinhibited conformation, similar to the N-WASP biosensor (Lorenz et al., Curr. Biol., 2004). Following transfection into COS-7 cells, the activation of WASPbs induced by constitutively-active Cdc42 (Q61L) was inhibited in the presence of the WASP inhibitor wiskostatin, indicating that changes in FRET observed accurately reported changes in WASPbs conformation and activity. WASPbs was expressed in the murine macrophage RAW/LR5 cell line and its activation was observed in response to multiple chemoattractants such as CSF-1, SDF-1 and fractalkine. In response to an upshift of CSF-1 concentration, WASPbs activation was detected globally as early as 30 sec and remained sustained in protrusive regions at later times. Confirmation that the WASPbs reflected activation of endogenous WASP was obtained using conformation-sensitive antibodies that only recognize the open (and therefore active) conformation of WASP and N-WASP. It has been speculated that following CSF-1 treatment PI 3-kinase activates guanine nucleotide exchange factors which in turn activate the Rho GTPases Rac and Cdc42. Since Cdc42 is a known activator of WASP, we determined whether PI 3-kinase and Cdc42 were required for CSF-1-stimulated WASP activation. WASPbs expressing RAW/LR5 cells either co-expressing a dominant-negative Cdc42 mutant (N17) or treated with the PI 3-kinase inhibitor wortmannin, showed reduced WASPbs activation following CSF-1 addition as compared to control cells. These results indicate that WASP activation downstream of the CSF-1R is PI 3-kinase and Cdc42 dependent.

2446

**Rho and its Effector Diaphanous Regulate Adherens Junctions**

C. Homem, R. B. Hauser, M. Peifer; Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

The coordinated tissue movements and cell shape changes that occur during morphogenesis require that cell-cell adhesion and its link to the actin cytoskeleton be very dynamic. In epithelial cells, cell-cell adhesion is mediated through Adherens Junctions (AJs), in which cadherins link cells by homophylic adhesion and in turn link to the actin cytoskeleton via α and β catenin. We are interested in molecules that may regulate the actin cytoskeleton at AJs. Recent findings suggest that formins are good candidates to be regulators of actin polymerization at sites of cell-cell adhesion. Previous studies in cultured mammalian cells have shown that formins interact with α-catenin and that formin function is important for the stability of cell contacts. Studies in *Drosophila* showed that Rho GTPase, a known regulator of Diaphanous, a *Drosophila* formin, is a regulator of AJs, although the mechanism of this regulation is not yet understood. To try to understand how are AJs dynamically regulated we are looking at how these actin regulators modify AJs during embryonic *Drosophila* development. Our hypothesis is that Rho regulates AJs through its effect on the actin cytoskeleton via Diaphanous (Dia). In our study we have found that *dia* co-localizes with AJs throughout much of *Drosophila* development. Further, we have seen that Dia interacts genetically with AJs components. To assess Dia's role during morphogenesis we are using a constitutive

active form of Dia. At dorsal closure two epithelial sheets migrate over the amnioserosal cells until both sheets meet and fuse. During this process the leading edge epithelium cells elongate and the amnioserosal cells constrict apically. Additional force is provided by actin-rich membrane protrusions. Initial results suggest that Dia activity has to be tightly regulated during dorsal closure in order for this each of these events to occur normally.

2447

### Function and Regulation of Fascin in Human Colonic Epithelial Cells

Y. Hashimoto, D. W. Loftis, J. C. Adams; Cell Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH

Fascin is an actin-bundling protein with roles in the assembly of cell protrusions that function in cell motility. Fascin is low or absent in normal epithelial cells, but is strongly expressed in human carcinomas from multiple tissues, where it is associated with poor prognosis (reviewed in Int. J. Biochem. Cell Biol. 37, 1787-1804). We hypothesise that fascin contributes to tumor cell migration through actin-bundling and through complexing of S39-phosphorylated fascin with protein kinase C (PKC) (see IJBCB 37, 1787-1804). A second objective is to understand how fascin becomes up-regulated in carcinoma cells. GFP-fascin and two point mutants, GFP-fascinS39A and GFP-fascinS39D, were over-expressed in human colonic epithelial SW1222 cells that lack endogenous fascin and clonal cell lines derived. All lines attached to collagen IV, fibronectin and laminin, but the wildtype or S39A-fascin-expressing lines were morphologically distinct with thick actin protrusions. Proliferation in tissue culture was equivalent in all lines. The major conventional PKC in colon epithelial cells is PKCgamma and we have demonstrated fascin binding to the C1 region of the regulatory domain. Human fascin promoter activity was compared in SW1222 and SW480 (a fascin-positive human colon carcinoma line), using a luciferase reporter assay. A 3.1kb genomic region upstream of the transcriptional start site significantly activated reporter expression in SW480 cells but had negligible activity in SW1222 cells. In contrast, a 1. 6kb genomic region upstream of the transcriptional start site (also negligible in SW1222 cells) had twice the activity of 3.1kb in SW480 cells. We conclude that the mechanism of fascin up-regulation in colon carcinoma cells may involve release from transcriptional repression that acts in the region -3.1kb to -1.6kb and that, once up-regulated, fascin contributes to altered tumor cell signaling and migration.

2448

### Involvement of PI(4,5)P2 in the Initial Activation of Cofilin During Carcinoma Cell Motility and Chemotaxis

X. Song, X. Chen, G. Mouneimne, J. S. Condeelis, R. J. Eddy; Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

The formation of cellular protrusions such as lamellipodia and invadopodia in response to EGF stimulation of mammary carcinoma cells involves the spatial and temporal control of actin dynamics. Cofilin, an actin severing protein, has been implicated in the initial steps leading to lamellipod protrusion and directional migration. In contrast to neutrophils, cofilin is initially activated following EGF stimulation of carcinoma cells by a mechanism that does not involve serine 3 dephosphorylation. The lipid second messenger PI(4,5)P2 has been proposed to integrate actin and membrane dynamics by regulating several actin-modulating proteins including cofilin. Furthermore, the initial peak of cofilin-dependent actin nucleation activity is diminished in the presence of the PLC inhibitor, U-73122. Although previous studies demonstrated that purified cofilin binds to PI(4,5)P2 micelles resulting in an inhibition of its actin severing activity, little is know about the interaction of cofilin with PI(4,5)P2 in cells. We observed that while the activites of cofilin and PLC increase within 60 sec after EGF stimulation, membrane-associated PI(4,5)P2 levels measured by GFP-PH(PLCdelta1) or a PI(4,5)P2-specific antibody decreases by half. This decrease in PI(4,5)P2 is accompanied by a reduction cofilin-PI(4,5)P2 colocalization at the membrane. Since PI(4,5)P2 is concentrated in cholesterol-dependent lipid microdomains (rafts), we investigated the association of cofilin with PI(4,5)P2 in rafts by TX-100 extraction followed by density gradient centrifugation. A fraction of the total cellular cofilin co-purified with lipid rafts. Preincubation of cells with methyl-beta-cyclodextrin prior to raft isolation reduced the levels of raft-associated cofilin significantly. Based on these observations, we propose the initial activation of cofilin may involve the release of a pool of raft-associated cofilin through activation of PLC during EGF-elicited protrusive events resulting in the first transient of action polymerization that determines the cell direction during chemotaxis.

## Cytoskeletal Organization II (2449-2472)

2449

### Analysis of the *Caenorhabditis Elegans* Septin Complex Indicates that Septin Filaments Have No Polarity

C. M. John,[1] D. J. Fitzgerald,[2] M. Faty,[1] H. Jawhari,[3] F. K. Winkler,[3] Y. Barral,[1] M. O. Steinmetz[3]; [1]Institute of Biochemistry, Swiss Federal Institute of Technology, Zuerich, Switzerland, [2]Institute of Molecular Biology and Biophysics, Swiss Federal Institute of Technology, Zuerich, Switzerland, [3]Structural Biology, Paul-Scherrer-Institute, Villigen PSI, Switzerland

Septins are conserved GTPases involved in cytokinesis and cell polarity. In budding yeast, where they were first discovered, septins form a ring at the bud neck. At the ultrastructural level, this ring is formed of the so-called 10 nm neck filaments, which are closely associated with the plasma membrane. The results of many studies indicate that the neck filaments are formed by polymerization of septin subunits into ordered arrays. Whether septin filaments have any polarity is not known. We investigated the molecular organization of septin complexes to determine whether septin filaments show any polarity. We chose to study the *Caenorhabditis elegans* septins Unc59 and Unc61 since they form the simplest septin system currently known. Unc59 and Unc61 both share the three conserved septin domains: a phospholipid binding domain directly N-terminal of a GTPase domain which is followed by a predicted coiled-coil region. To reconstitute a soluble and functional *C.elegans* septin complex, Unc59 and Unc61 were co-expressed in *E.coli* and insect cells and affinity purified. By biochemical and biophysical means we show that Unc59 and Unc61 form a heterodimer that assembles tail-to-tail into a dumbbell shaped heterotetramer. Septin coiled-coil domains both stabilize the heterodimer and participate in the assembly of the heterotetramer. The heterotetramer forms the polymerization-competent subunit of septin filaments. Filaments elongate along the long axis of the tetramer, while lateral interactions mediate the formation of filament bundles and sheets. The tail-to-tail architecture of the tetramer suggests that Unc59 and Unc61 form a non-polar complex. This architecture is consistent with that of core septin complexes of yeast. Our data provide a first model to understand the formation of more elaborate septin complexes, and suggest that septin filaments in general have no polarity.

2450

**Rhotekin, a Small GTPase Rho Effector, Regulates Septin Filament Structures in Rat REF52 Cells**

K. Nagata,[1] H. Ito,[1] I. Iwamoto,[1] R. Morishita,[1] Y. Nozawa,[2] S. Narumiya,[3] T. Asano[1]; [1]Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Japan, [2]Gifu International Institute of Biotechnology, Gifu, Japan, [3]Pharmacology, Kyoto University Faculty of Medicine, Kyoto, Japan

There is growing evidence for cross-talk between septin filaments and actin cytoskeleton which is regulated by Rho family of GTPases. Here we show that active Rho disrupted septin filament structures in REF52 fibroblast cells. Among Rho effector molecules tested, Rhotekin showed morphological changes of septin filaments similar to those by activated Rho. The center region of Rhotekin was sufficient for the septin reorganization in the cells, and likely to interact indirectly with the C-terminal half of Sept9v3, where a GTPase domain is located. Rhotekin and Sept9v3 are co-localized in perinuclear regions in serum-starved REF52 cells while they redistributed along with actin stress fibers upon stimulation with lysophosphatidic acid (LPA). In neuroblastoma Nuero2a cells, Rhotekin and Sept9v3 were enriched in the tip of neurites, a location where cortical actin reorganization is induced upon stimulation with LPA. Taken together, we propose that Rhotekin is a novel regulator organizing mammalian septin structures and provide a new link between the septin and Rho-signaling.

2451

**The Structural Dynamics of the Yeast Septin Polymer Determined by *in vivo* FRET Microscopy**

K. S. Thorn, M. Sheff; Bauer Center for Genomics Research, Harvard University, Cambridge, MA

The septins are a group of proteins, conserved from yeast to humans, that play important roles in cytokinesis, membrane compartmentalization, and exocytosis. These functions depend on their polymerization into filaments; however, the structure of these filaments is currently unknown. Here, we use fluorescence resonance energy transfer (FRET) microscopy to measure the distances between individual components of the septin filament in live *S. cerevisiae*, in both G1 and metaphase cells and in cells deleted for known regulators of septin function. To make these measurements, the five vegetative yeast septins (Cdc3, Cdc10, Cdc11, Cdc12, and Shs1) were tagged at their C-termini with CFP or YFP. These measurements identify a number of structural changes in the septin complex that occur between G1 and metaphase. Deletion of the kinases Cla4 or Gin4, or the septin Shs1, blocks one of these structural changes, a shortening of the distance between Cdc10 and Cdc11 in metaphase, but introduces a number of constitutive changes to the structure of the septin complex. These data uniquely determine a structural model of the monomeric septin complex and of the interfaces involved in septin polymerization. In this model the long axis of the septin complex is spanned by a heterodimer of Cdc3 and Cdc12 homodimers. Cdc10 and Cdc11 bind to the globular ends of Cdc3 and Cdc12, which interact with adjacent monomers in the end-to-end polymer. This model is supported by biochemical data on the septin complex. Our data further suggest that in G1 cells the septin complex is primarily present as individual linear polymers, and that the structural changes in the septins that are known to occur after bud-emergence result from side-to-side binding of these filaments.

2452

**Characterisation of SEPT9_v1 Protein Expression in Human Epithelial Cells**

P. L. Hyland, P. A. Hall, H. Russell; Centre for Cancer Research & Cell Biology, Queens Univesity Belfast, Belfast, United Kingdom

The expression of SEPT9, one of 13 known human septins, is perturbed in human neoplasia. While mutations have not been found, alterations in the level of SEPT9 transcripts are seen. In particular, changes in the levels of SEPT9_v1, v4 and v4* transcripts have been observed. Furthermore, we have shown that SEPT9_v4 can promote altered motility, perturb polarity and promote resistance to microtubule acting drugs. Our attention has therefore turned to the properties of SEPT9_v1 and we have constructed a model system that mimics the over-expression of this transcript in epithelial neoplasia. To this end we have over-expressed SEPT9_v1 in MCF7 cells both transiently and stably. We have also expressed point mutants that represent both gain of function (G308V) and dominant negative (loss of function; S312N) forms. Immunofluorescence showed both cytoplasmic and nuclear localization for wild-type SEPT9_v1 ranging from filamentous to particulate in nature, similar to that seen with endogenous SEPT9_v1 protein. In contrast, neither mutant was associated with filaments. Biochemical fractionation studies showed that both endogenous SEPT9_v1 and epitope-tagged wild-type SEPT9_v1 was present in cytosol, membrane, cytoskeleton and nuclear fractions. The latter observation is supported by the presence of a bipartite-NLS in the N-terminus of SEPT9-v1. In contrast, epitope-tagged mutant SEPT9_v1 (G308V and S312N) protein was not detected in nuclear fractions. Expression of SEPT9_v1 S312N induced prominent microtubule containing neurite-like extensions, not seen in the wild type or G308V expressing cells. We observe that the expression of SEPT9_v1 alters the adhesive properties of epithelial cells paralleling these morphological changes. Moreover the growth of SEPT9_v1 S312N expressing cells differ from that of other cells with a propensity to form multilayered colonies. These studies provide possible insights into the role of deregulated SEPT9_v1 expression in neoplasia.

2453

**Characterisation of the Functional Domains of SEPT9**

N. L. Pentland, P. L. Hyland, C. J. Scott, H. Russell, P. A. Hall; Centre for Cancer Research & Cell Biology, Queens University Belfast, Belfast, United Kingdom

Altered expression of SEPT9 is a common occurrence in neoplasia and recent data from our group indicates that over-expression of SEPT9_v4 promotes cell motility, perturbs polarity and can induce resistance to microtubule interacting drugs. We wish to understand the domains of SEPT9 that can confer such properties and determine the functional elements that contribute to SEPT9 interactions both with itself and other proteins. In addition to all wild type forms of SEPT9 (v1,v2,v3,v4 & v5), we have generated a complex series of nested deletion mutants as epitope tagged constructs, including: the N terminal variants (v1 residue 1-164; v2 residue 1-157; v3 residue 1-146); the unique region of SEPT9_v1 (residue 1-25); the common region of the N terminal forms (v1 residue 26-164); the N terminus of SEPT9_v4 (v4 residue 1-87); the GTP binding moiety, with (v5 residue1-220) and without the polybasic region (v5 residue 51-220); the GTP binding region plus the septin unique region (v5 residue 51-335) and the septin unique region (v5 residue 221-335). We have employed these constructs to examine homotypic and heterotypic interactions of the various domains in cell based assays by immunoprecipitation as well as confirming interactions using purified proteins. We observe evidence for homotypic interaction of SEPT9_v1 and this appears to be mediated by sequences in both the GTP binding domain as well as via sequences in

the N terminus. SEPT9_v2 and v3 are almost identical to SEPT9_v1, yet do not appear to show an interaction with SEPT9_v1 mediated by the N terminal moieties. Other immunoprecipitation experiments show that SEPT9_v5 can participate in homotypic interactions. In other experiments we are investigating the localisation of the various transgene encoded proteins and examining their functional consequences. Based upon this we can propose a model of how SEPT9 complexes can be formed.

2454

**Role of Lipids in the Assembly of the Central Spindle and Actin Ring during Cytokinesis**

I. E. Szafer-Glusman, M. Fuller; Developmental Biology, Stanford School of Medicine, Stanford, CA

Although it has long been recognized that a contractile actin ring positioned at the equator of dividing cells, and an equatorial bundle of antiparallel microtubules (central spindle) actively participate in cytokinesis, the mechanisms that regulate and mediate assembly of these critical structures, as well as their inter-relationship remain largely unknown. In a large genetic screen for cytokinesis defects in Drosophila male germ cells, mutations that affect cytokinesis in meiosis were identified (Giansanti et al., 2004). One of these mutant genes, *bond*, fails to assemble a central spindle and the actin ring during early anaphase, but allows for the correct localization of myosin and anillin to the cell equator. Molecular cloning and sequencing analysis of *bond* alleles revealed mutations in a novel gene that belongs to the family of Elovl genes conserved from yeast to mammals. Elovl proteins are involved in the elongation of very long chain fatty acids, precursors of complex membrane sphingolipids. In accordance with these findings, inhibition of sphingolipid synthesis in cultured S2 cells induced cytokinesis failure, suggesting that *bond* and *bond*-dependent lipid domains may function in the assembly and organization of cortical F-actin and/or microtubules during cytokinesis. Perhaps these putative lipid domains serve as scaffolds for the recruitment of factors that may function in the regulation or assembly of microtubules or F-actin to the membranes.

2455

**Role of Microtubules and Tea1p in the Regulation of Cell Polarity in *Schizosaccharomyces pombe***

R. Basu,[1] D. Khodjakov,[2,3] S. G. Martin,[1] F. Chang[1,2]; [1]Microbiology, Columbia University, New York, NY, [2]Marine Biology Laboratory, Woods Hole, MA, [3]Columbia High School, East Greenbush, NY

Growth in the fission yeast *Schizosaccharomyces pombe* occurs by extension of the ends of its cylindrical structure. Microtubules have been implicated in establishing bipolar growth in a process known as new end take off (NETO). Direct observations show that microtubule plus ends transport the polarity factors tea1p and tea4p and deliver them to the cell tips. These factors subsequently recruit the formin-actin machinery responsible for cell growth. In this study we evaluate if microtubules are indeed necessary for the establishment of bipolar growth in fission yeast. FRAP studies using tea1-GFP showed that tea1p is delivered to the cell tips with a $t_{1/2}$ of about 5 mins. Interestingly, similar dynamics occurred even in cells treated with the microtubule-depolymerizing drug, MBC, showing that tea1p can dock in the absence of microtubules. We next monitored cellular growth patterns using time-lapse microscopy. In wildtype cells treated with MBC at the beginning of the cell cycle, most cells exhibited monopolar growth (NETO defective), but about 17% of the cells still grew in a bipolar fashion. In contrast, *tea1* mutant cells all exhibited monopolar growth both in the absence and presence of MBC. These data suggest that microtubules do contribute to NETO, but also that tea1p can dock and function even in the absence of microtubules. Current experiments focus on the contribution of other tea1-associated proteins on the microtubule-independent function of tea1p.

2456

**Organization and Force Generation of Interphase Microtubules in Fission Yeast**

L. Krueger,[1] C. Pantoga,[1] D. Bhatt,[2] D. Foethke,[3,1] M. Dogterom,[4,1] F. Nedelec,[3,1] P. Tran,[2] M. Janson[2,1]; [1]Physiology Course, Marine Biological Laboratory, Woods Hole, MA, [2]Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA, [3]Cell Biology and Biophysics Program, EMBL, Heidelberg, Germany, [4]FOM Institute AMOLF, Amsterdam, The Netherlands

Interphase fission yeast cells organize 2 to 5 microtubule bundles that run along the long axis of cells. Each bundle contains two or more antiparallel microtubules that are overlapped and bound to the nuclear envelope at their minus ends, while the plus ends grow toward the cell ends. At the cell poles polymerization creates pushing forces that position the nucleus towards the cell middle. Microtubule bundles also deliver polarity factors to the cell cortex and thereby restrict cell growth to the cell poles. To perform these symmetry-maintaining roles it seems important that multiple bundles are well dispersed over the nuclear envelope and that the growth dynamics of microtubules is regulated at the cell cortex. This summer at the Woods Hole physiology course we investigated possible regulatory mechanisms. First, we observed that bundles in wild type cells are more regularly spaced over the nuclear envelope than would be expected by random placement. We used the software Cytosim to simulate the process of bundle formation and organization. We investigated whether a factor that selectively bundles anti-parallel microtubules could explain the observed bundle distribution. Second, we investigated how microtubule growth dynamics responds to forces generated in contact with the cell wall. We inferred growth velocities and forces from the buckling of single microtubules in contact with cell walls. For practical purposes we used round mutant cells (mor2-phenotype) that lacked the microtubule bundling protein ase1p. Our analysis shows that microtubule growth in yeast may be optimized for force generation as the growth velocity was decreased less by force than previously observed for microtubules polymerized from pure tubulin.

2457

**Coordination of Microtubule and Actin Functions is Essential for Rapid Hyphal Growth of *Aspergillus Nidulans***

T. Horio,[1] X. Dou,[2] S. A. Osmani,[2] B. R. Oakley[2]; [1]Institute of Health Biosciences, The University of Tokushima, Tokushima, Japan, [2]Department of Molecular Genetics, Ohio State University, Columbus, OH

Polarized growth is observed in many organisms and cell types. Cytoskeletal components are known to play important roles in this process. Tip growth of filamentous fungi serves as a model for polarized growth. Previously, we have reported that microtubules play an essential role in rapid hyphal growth of *A. nidulans* (MBC, 2005, 16, 918-926). The actin cytoskeleton is known to localize near the tip of the cell and play an essential role in the deposition of wall materials at the growing tip. To investigate the coordination of actin and microtubules in this process, we observed rapidly growing hyphae of a strain expressing GFP-labeled actin by time-lapse microscopy. In rapidly growing hyphae, actin localized to the

subapical region in a ring of plaques. The ring moved forward following the tip growth keeping even spacing from the tip. Treating hyphae with the anti-microtubule agent benomyl resulted in a vast reduction of the growth rate. The actin plaques dispersed through the cytoplasm as growth slowed. In about 40% of the hyphae, actin plaques failed to reorganize to any specific region throughout the observation period. However, in other hyphae, actin plaques re-accumulated either at the tip or in a medial region of the hyphal wall and slow growth resumed at the site of actin accumulation. After washing out benomyl, rapid tip growth resumed prior to the reorganization of acting plaques. The actin plaques became reorganized as the tip growth rate accelerated. Our results reconfirm the idea that functional actin is essential for the tip growth of filamentous fungi and indicate that coordination of actin and microtubules is required for organization of subapical actin plaques that is required for rapid and steady hyphal tip growth.

2458

**Contribution of Actin-Binding Protein Filamin A to the Process of Stress Fiber Formation in Nonmuscle Cells**

K. Kato,[1] H. Haga,[1] K. Ohashi,[2] T. Ito,[3] K. Kawabata[1]; [1]Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Japan, [2]Department of Biology, Faculty of Science, Chiba University, Chiba, Japan, [3]Division of Biological Sciences, Graduate School of Science, Kyoto University, Kyoto, Japan

Filamin A (FLNa) is one of the actin-binding proteins that cross-links actin filaments and makes 3D actin networks at lamellipodia and cell cortices. FLNa is also expressed strongly at stress fibers in nonmuscle cells, indicating FLNa might contribute to some functions of stress fibers. We found previously that expression of FLNa led to an increase of intracellular tension resulted from the formation of stress fibers at dorsal cortex. In this study, to clarify the process of FLNa-dependent stress fiber formation, we observed spatial and temporal variations of stress fibers in FLNa-deficient human melanoma cells (M2 cells) and M2 derived cell-line expressing FLNa (A7 cells) by using immunofluorescent and GFP techniques. In mature A7 cells attaching to glass substrates, stress fibers were formed at the dorsal cortex, whereas stress fibers were not observed at the dorsal of M2 cells. To observe the process of the stress fiber formation, time-lapse imaging of GFP-tagged myosin regulatory light chain in spreading cells was performed using confocal laser scanning microscopy. In A7 cells, polygonal structures at basal and ring-like structures at approximately 4 μm above the base were observed at early stage of the stress fiber formation. Once cells elongated, the stress fibers at the dorsal cortex in A7 cells aligned with the axis of the cell elongation. We also examined the spatial distribution of FLNa in the stress fibers. FLNa was localized alternatively with myosin II in the stress fibers, as well as in the muscle fiber. All these results suggest that FLNa might control the spatial distribution of stress fibers.

2459

**Shear-Induced Alterations in Cell Morphology and Stress Fiber Reorganization in Osteoblast and Osteocytes**

S. M. Ponik, J. Triplett, R. O'Riley, F. Pavalko; Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN

Cells subjected to fluid shear stress undergo significant architectural and biochemical changes. Our lab and others previously demonstrated increased actin stress fiber formation in osteoblasts subjected to unidirectional shear stress. To further evaluate the morphologic response of bone cells to different shear profiles, we analyzed changes in cell elongation, long axis alignment, and stress fiber organization in MC3T3 osteoblasts and MLO-Y4 osteocytes subjected to unidirectional and oscillatory shear stress. Unlike what has been reported for endothelial cells, we found that the shear-induced increase in actin stress fiber formation in MC3T3 osteoblasts was not accompanied by cellular realignment in the direction of flow, regardless of the type of shear stress. However, the percent of MC3T3 osteoblasts with significant elongation increased from 35% in static culture to 51% and 85% after 24 hours of oscillatory and unidirectional shear stress, respectively. Analysis of MLO osteocytes revealed that unlike osteoblasts neither cell elongation nor long axis alignment is altered in response to unidirectional or oscillatory shear stress for up to 24 hours. We did find differences in the temporal response of stress fiber formation in osteoblasts treated with either unidirectional or oscillatory shear. As reported previously, immunofluorescence analysis showed a dramatic reorganization of stress fibers in MC3T3 osteoblasts after just 1 hour of unidirectional shear. The same rearrangement of stress fibers did not occur in osteoblasts subjected to oscillatory shear until after 24 hours of stimulation. Additionally, only a modest reorganization of actin microfilaments was observed in MLO osteocytes after 24 hours of unidirectional flow. While shear stress is a potent stimulus for bone cell metabolism, these data suggest inherent differences in the architectural response of osteoblasts and osteocytes to unidirectional and oscillatory shear stress.

2460

**Mechanics and Shape Contributions of Single Stress Fibers in Living Cells**

S. Kumar,[1] I. Z. Maxwell,[2] A. Heisterkamp,[3] T. P. Lele,[4] T. R. Polte,[4] E. Mazur,[2] D. E. Ingber[4]; [1]Department of Bioengineering, University of California, Berkeley, Berkeley, CA, [2]Division of Engineering and Applied Sciences, Harvard University, Cambridge, MA, [3]Lazer Zentrum Hannover, Hannover, Germany, [4]Vascular Biology Program, Children's Hospital Boston and Harvard Medical School, Boston, MA

Cells change their form and function by assembling actin stress fibers at their base and exerting traction forces on their extracellular matrix (ECM) adhesions. It is widely assumed that individual stress fibers are actively tensed by the action of actomyosin motors and that they function as elastic cables that reinforce the basal portion of the cytoskeleton; however, these principles have not been directly tested in living cells, and their significance for overall cell shape control is poorly understood. Here we combine a femtosecond laser nanoscissor, traction force microscopy, and fluorescence photobleaching methods to show that each stress fiber in a living cell behaves as a viscoelastic cable that is both actively tensed by actomyosin motors and passively prestressed through ECM adhesions. By using femtosecond pulses, our nanoscissor is capable of *selectively incising and puncturing holes in individual stress fibers in living cells* with submicron precision and without compromising the plasma membrane. Surprisingly, stress fibers continue to support substantial tensile loads following inhibition of actomyosin contraction. Moreover, the degree to which a single stress fiber contributes to cell shape depends on the rigidity of the ECM. In cells cultured on rigid glass ECMs, disruption of multiple parallel fibers does not significantly alter cell shape. Conversely, in cells cultured on compliant ECM substrates, disruption of a single stress fiber transfers forces to multiple distant adhesion sites, induces cytoskeletal rearrangements many microns away from the site of incision, and changes the shape of the whole cell. In addition to revealing fundamental insight into the mechanical properties and cell-shape contributions of individual actomyosin stress fiber bundles, the technologies described here offer a novel experimental framework with which to spatially map the cytoskeletal mechanics of living cells on the nanoscale.

2461

**Self-organization and Computational Modeling of the Basal Microtubule Network in Polarized Epithelial Cells**

A. R. Reilein, S. Yamada, W. J. Nelson; Molecular and Cellular Physiology, Stanford University, Stanford, CA

Centrosome-derived microtubule arrays have been well-studied, but less is known about the formation and dynamics of non-centrosomal microtubule networks in cells. We examined microtubule networks on the basal cortex of polarized epithelial cells experimentally, and with a stochastic simulation. By examining microtubule dynamics in basal cytoplasts prepared from polarized cells, we found that unlike non-centrosomal networks that require microtubule treadmilling or microtubule motor proteins for organization, this network arises from microtubule-microtubule and microtubule-cell cortex interactions that include Adenomatous polyposis coli (APC). Clathrin-containing and LysoTracker Red-labeled vesicles move bi-directionally along the microtubules, indicating that the basal microtubules are functional in endocytosis. Although microtubules on the basal cortex have an apparently random orientation, the network is self-organized. Stochastic simulation of de novo network formation shows that without any stabilization points the microtubule network does not reach a steady-state configuration. However, in the presence of randomly-generated cortical attachment points (mimicking APC) and microtubule end-to-side interactions, both simulated by an increased microtubule rescue frequency, microtubules cluster over the cortical spots and reach a steady state pattern. Together, these results show a mechanism by which mixed-polarity microtubules can self-organize on the cell cortex to form a functional microtubule network.

2462

**Polymer Physics of the Cytoskeleton**

B. D. Hoffman, K. A. Miranda, G. Massiera, J. C. Crocker; Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA

The importance of mechanics and force to biological processes is increasingly appreciated and affects behaviors as distinct as cell motility, morphogenesis, and differentiation. In this work we use four microrheological techniques to measure cellular mechanical properties in a variety of cell types. In combination, these techniques have the novel ability of probing cell mechanics over a wide range of frequency, length scale, geometry and driving (applied torques or innate thermal driving) in a single cell. We find a remarkably simple, but rich, consensus behaviour that, when interpreted with recent advances in polymer physics, shows for the first time that cells behave as a network of semi-flexible polymers, like actin or intermediate filaments, cross-linked by extensible proteins with unfoldable domains, like filamin or actinin. Furthermore a physical mechanism explaining the origins of these properties should help to identified mechanisms of mechanotransduction. Therefore we developed a model of cytoskeletal mechanics based on thermally activated, forced unfolding of protein cross-links in a stressed polymer gel. We find these unfolding events produce an emergent, nearly exponential distribution of cross-link tension. Such tension distributions readily reproduce the recently observed power-law form of cells' dynamic shear moduli as well as power-law creep response. Furthermore the primary determinant of the cellular mechanical response should be the strength of bonds holding together the cross-link domains. Unlike some existing mechanical models the model has a clear connection to cell signaling, as unfolded domains should be readily detectable biochemically. Interestingly, the number of unfolded domains in the simulations is a monotonic function of the strain and shows no hystersis. This leads to novel hypothetical mechanisms of cell shape sensing, compliance sensing, and mechanotransduction as the cell could "measure" the number of these domains to infer the strain in its local environment.

2463

**Image-Based Identification of Small-Molecule Inhibitors of Yeast Pathogen Morphogenesis**

T. Lila,[1] P. Chua,[1] C. Vacin,[1] S. Collibee,[1] D. Clarke,[1] D. Roof,[1] B. Morgan,[1] M. Maxon,[2] D. Pierce,[1] J. Finer[1]; [1]Cytokinetics Inc., South San Francisco, CA, [2]Institute for Regenerative Medicine, San Francisco, CA

Yeast cells can respond to perturbation of the cell cycle or cytoskeleton with distinctive changes in extracellular and nuclear morphologies. To identify compounds that alter the morphology of *Candida albicans*, we conducted an automated fluorescence-microscopic screen of cells treated with a library of 6800 compounds known to be *C. albicans* growth inhibitors. 154 compounds causing diverse morphological phenotypes were identified, including one structural class that induces an isotropic growth phenotype characteristic of defects in polarization of the actin cytoskeleton. A survey of structual analogs in this class revealed members that lack appreciable human cell toxicity and have low micromolar morphogenesis-inhibitory potencies against *C. albicans, C. glabrata, and C. tropicalis* species. Inhibition of morphogenesis by compounds in the series is associated with protracted growth inhibition, making the compounds of interest as potential broad spectrum antifungal agents.

2464

**Interaction of *Plasmodium falciparum* Erythrocyte Membrane Protein 3 (PfEMP3) with Red Cell Membrane Skeleton**

X. Pei; Red Cell Physiology, New York Blood Center, New York, NY

*Plasmodium falciparum* erythrocyte membrane protein 3 (PfEMP3) is one of a large group of malaria proteins which are exported into red blood cytoplasm during parasite development. It has been previously shown that PfEMP3 contributes both to cytoadherence and to rigidity of malaria-infected red blood cells. Nonionic detergent solubility experiments strongly suggested a linkage between PfEMP3 and the red blood cell membrane skeleton. However the nature of interaction of PfEMP3 with red cell membrane skeletal proteins remains largely unknown. In the present study, we show that PfEMP3 binds to both spectrin and protein 4.1R. The binding sites in PfEMP3 for both spectrin and 4.1R were mapped to a 60 amino acid region (FIa1: residues 38 to 97). The FIa1 fragment bound only to one (α 17-C) of nine spectrin fragments which encompass the entire α and β spectrin molecules. The FIa1 binding site within the α 17-C fragment was further localized to repeat 18 and EF hands. Similarly, of the four structural domains of 4.1R, only the N-terminal 30 kDa membrane binding domain was shown to interact with PfEMP3. Furthermore, we documented that the interaction of either full length spectrin or 4.1R to PfEMP3 could be specifically inhibited by the α 17-C spectrin fragment and the 30 kDa membrane binding domain of 4.1R respectively. Detailed molecular characterization of interactions between malaria exported proteins with red cell membrane skeletal proteins should provide insights into mechanisms by which malarial proteins modify red blood cell membrane properties.

2465

**New Cytoskeletal Structures Used to Build a Parasite**

K. Hu,[1] S. Suravajjala,[2] D. S. Roos,[2] J. M. Murray[3]; [1]Dept. of Cell Biology, Scripps Research Institute, La Jolla, CA, [2]Dept. of Biology, University of Pennsylvania, Philadelphia, PA, [3]Dept. of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

*Toxoplasma gondii* belongs to the phylum *Apicomplexa*, which includes 5,000 species of protozoan parasites, including many important human and veterinary pathogens with remarkable cytoskeletons. Central to both invasion and proliferation of *Toxoplasma* is the apical complex, a group of apical cytoskeletal and membrane-bound organelles built around the "conoid", an assembly of 14 spirally arranged novel tubulin polymers that is actively motile during invasion (Hu et al., 2002). In this study, we characterize the localization and dynamics of several apical complex components previously identified in a proteomics screen (Hu et al. 2005). These proteins are not only new markers to sub-compartments within the apical complex, but also define new compartments within the parasite scaffold. In particular, TgDynein Light Chain (TgDLC) concentrates at the ends of the conoid fibers and the anterior portion of the cortical microtubules. TgCentrinII, labels the centrioles , the pre-conoidal rings and 5-6 peripheral rings. Interestingly, the peripheral centrin rings occupy the border of the cap of GFP-TgDLC at the apical end of the parasite, indicating that the molecular composition of anterior and posterior cortical scaffold is distinct. TgMORN1 is a new component of the apical complex and the spindle pole, which in adult parasites also fills the basal gap of the IMC filament network. In dividing cells, TgMORN1 caps the ends of the growing daughter scaffolds. Using time-lapse microscopy, we established the sequence of recruitment of these proteins into the cytoskeleton during daughter scaffold formation. In addition, we studied the distribution of these proteins upon disrupting cell division using microtubule inhibitors to further understand the biogenesis of the new membrane/cytoskeletal compartments that these proteins have defined. Our results yield new insights into the roles of these apical complex components in the construction of the parasite membrane-cytoskeleton scaffold.

2466

**Bacteria Mechanics: Defining the Role of the Cytoskeleton**

O. Esue,[1] Y. Tseng,[2] D. Wirtz[1]; [1]Chemical and Biomolecular Engineering, Johns hopkins University, Baltimore, MD, [2]Chemical Engineering, University of Florida, Gainesville, FL

MreB, FtsZ, and crescentin are highly homologous to eukaryotic actin, tubulin, and intermediate filaments respectively. Depletion of genes coding for MreB and FtsZ cause defects in cell morphology, division, and chromosome segregation, while depletion of crescentin causes *Caulobacter Crescentus* to loose its crescent shape. These results imply both biochemical and mechanical roles for these eukaryotic cytoskeleton protein homologs in bacteria. We use a cone and plate strain controlled rheometer and electron microscopy to study the gelation, structural, and rheological properties of these proteins and establish their structural significance in the cell. We find that MreB, crescentin, and FtsZ form filaments that gel extremely rapidly, with highly elastic networks within physiological time scales (~ minutes), much faster than other major dynamic cytoskeletal filaments in eukaryotic cells, including microtubules, actin and, vimentin. These findings suggest a mechanical role for MreB in cell shape, chromosome segregation, and possible intracellular transport; and crescentin filaments in shaping *C. Crescentus* cells; while FtsZ filaments have the toughness to provide strong mechanical support for the maintenance and circumferential constriction of the bacterial Z-ring. These studies may form a molecular basis for the development of the new generation of antibiotics.

2467

**EhPAK2, a Novel p21-activated Kinase, is Required for Cytokinesis, Capping and Collagen Invasion in *Entamoeba histolytica***

L. E. Arias-Romero, M. J. Almaraz-Barrera, J. D. Diaz-Valencia, M. A. Vargas-Mejia; Biomedicina Molecular, CINVESTAV-IPN, Mexico City, Mexico

Rho GTPases are a family of molecular switches that control fundamental cellular processes in eukaryotic cells and they are known principally for their role in regulating the actin cytoskeleton. Rho GTPases are low-molecular weight proteins that cycle between two conformational states: an inactive GDP-bound form and an active GTP-bound form. Binding of GTP activates Rho GTPases by inducing structural shifts that support association of effector molecules that transmit downstream signals. The p21-activated kinases (PAKs) were the first GTPases-regulated kinases to be identified. They are serine/threonine kinases that serve as important mediators of Rac and Cdc42 GTPases. The kinase activity of PAK has been implicated in proliferative signaling by growth factor receptor tyrosine kinases, as well as in morphogenic processes that control cell polarity and actin cytoskeleton organisation. Recently, a putative PAK has been described in *E. histolytica* and plays a role in the amoeba polarity, motility and phagocytosis of human red blood cells. The aim of this study is to describe the function of a novel p21-activated kinase, EhPAK2. Our results have shown that the CRIB domain of EhPAK2 was able to bind *in vitro* to the *E. histolytica* GTPases EhRacA, EhRacG and EhRho1, whereas the over expression of C-terminal kinase domain of EhPAK2 in amoeba trophozoites, caused severe defects in capping and cytokinesis as well as a significant reduction in amoeboid collagen invasion, suggesting an involvement of EhPAK2 in these cellular processes.

2468

**Simultaneous Analysis of Actin and Myosin II Reveals Three Modes of Contraction-Based Actin Organization in Migrating Epithelial Cells**

J. I. Lim, L. Ji, C. M. Waterman-Storer, G. Danuser; Cell Biology, The Scripps Research Institute, La Jolla, CA

Cells move by coupling forces generated by myosin II-based contraction in the actin cytoskeleton to the extracellular matrix. We previously used quantitative fluorescent speckle microscopy (qFSM) to define kinetically, kinematically and molecularly distinct actin-based modules mediating epithelial cell migration: the lamellipodium being the protrusive module and the lamella being the adhesive/contractile module. In the lamella, retrograde flow of actin powered by myosin II meets a myosin II- powered anterograde actin flow from the cell center in a region we termed the convergence zone. As hypothesized in the solation-contraction coupling model, contraction forces driving actin motion may promote the disassembly of actin filaments in the convergence zone. To test this hypothesis, we co-injected labeled actin with GFP-tagged myosin regulatory light chain (mRLC-eGFP) in PtK1 epithelial cells and imaged them by multispectral spinning disk confocal microscopy to investigate the role of actin and myosin in the convergence zone. qFSM image analysis tools have allowed us to generate spatiotemporal maps of actin polymerization and depolymerization and mRLC assembly and disassembly. We have additionally developed tools that allow correlation of the direction and speed of actin and mRLC-eGFP speckles. Using these tools we find that the flow of actin and mRLC are coupled in the lamella, whereas the absence of

mRLC (and hence, decoupling with actin) at the cell edge supports the actin-polymerization induced retrograde flow model in the lamellipodia. Our investigation of actin flow in the convergence zone provides evidence for three distinct modes of contraction-based actin organization associated with mRLC-eGFP : 1. “Compression” in which actin meshwork convergence is coupled to actin depolymerization; 2. Shear - in which contraction induces anti-parallel actin filament motion; 3. Bundling in which contraction induces actin bundle formation.

2469

**Abl Interactor 1 is Required for Bcr-Abl to Induce Actin Cytoskeleton Remodeling and Integrin Clustering in Hematopoietic Cells**

Y. Li, N. Clough, Z. Dai; Medicine/Medical Oncology, University of Colorado Health Sciences Center, Aurora, CO

The hematopoietic cells isolated from patients with Bcr-Abl-positive leukemia exhibit multiple abnormalities of cytoskeletal function. The molecular events leading to these abnormalities are not fully understood. Here we show that the expression of $p185^{Bcr\text{-}Abl}$ in hematopoietic cells induces tyrosine phosphorylation of Abl interactor (Abi) 1 and the membrane translocation of Abi 1/WAVE 2 complex. This correlates with a striking actin cytoskeleton remodeling; particularly, the induction of an actin-enriched structure located in membrane protrusion. Abi 1, Bcr-Abl, and WAVE 2 were found to co-localize with this abnormal F-actin structure. The proteins involved in cell adhesion, such as integrin, paxillin, and vinculin were also found in association with this actin-enriched structure. Blockade of the signal transduction from Bcr-Abl to Abi 1 by disrupting the interaction between the two proteins not only abolished the tyrosine phosphorylation of Abi 1 and membrane translocation of Abi-1/WAVE 2, but also abrogated the ability of $p185^{Bcr\text{-}Abl}$ to induce abnormal F-actin reorganization and integrin clustering. Moreover, expression of a mutant Abi 1 defective in binding to $p185^{Bcr\text{-}Abl}$ inhibited the Bcr-Abl-induced abnormal actin cytoskeleton remodeling and integrin clustering. These studies define Abi-1/WAVE 2 as a downstream pathway utilized by $p185^{Bcr\text{-}Abl}$ to induce abnormal interaction between integrin and cytoskeleton, and may help to explain how Bcr-Abl induces abnormal adhesion, migration, and homing of leukemic cells.

2470

**Regulation of Cell Migration and Spreading by the Ring Finger E3 Ligase Rnf5**

A. Delaunay, C. Didier, A. Bhoumik, K. Nakayama, Z. Ronai; Signal Transduction Program, The Burnham Institute, La Jolla, CA

RNF5 is a membrane anchored E3 ubiquitin ligase conserved from *C elegans* to Human. In the nematode, RNF-5 regulates the stability of a LIM domain protein involved in the formation of dense bodies and muscle attachment sites, structures that can be compared to mammalian focal adhesion (Broday et al., JCB 2004). In Mammals, one of RNF5 targets is the focal adhesion protein, paxillin, which coordinates cytoskeletal organization during cell migration and spreading. RNF5 overexpression leads to the ubiquitination-dependent exclusion of paxillin from focal adhesion and is associated with an inhibition of cell migration (Didier et al., Mol Cell Biol, 2003). To elucidate the physiological role of RNF5 in the control of paxillin dynamics and cell motility, we first monitored the endogenous localization of RNF5 in normal fibroblasts. RNF5 was found within the leading edge of migrating cells, as well as in specialized, ring shape Spreading Initiation Centers involved in the maturation of focal adhesion (de Hoog et al., Cell 2004). In both cases, RNF5 co-localized with a disorganized fraction of paxillin while in spread and/or static cells, where paxillin is fully organized in mature focal adhesions, RNF5 staining was rather low and diffuse. Consistent with these findings, MEFs obtained from *RNF5-/-* mice exhibited a delay in cell adhesion and spreading which is associated with a delay in the formation of focal adhesion. Impaired cytoskeleton organization was also found in *RNF5-/-* cells that were subjected to stress. These results suggest that RNF5 plays an important role in the control of focal adhesion maturation and/or turnover, as well as in cytoskeleton remodeling. Importantly, both RNF5 E3 ligase activity and membrane anchoring are required for RNF5’s function in cell adhesion and motility.

2471

**Dip Regulates mDia2-mediated Membrane Remodeling at the Leading Edge of Migrating Cells**

K. M. Eisenmann,[1] S. M. Kitchen,[1] H. A. Holman,[1] E. S. Harris,[2] H. N. Higgs,[2] A. S. Alberts[1]; [1]Laboratory of Cell Structure and Signal Integration, Van Andel Research Institute, Grand Rapids, MI, [2]Dartmouth University, Hanover, NH

WASP/N-WASp, which promote branched-nucleation of actin via Arp2/3, and the mammalian Diaphanous-related (mDia; mDia1-3) formins, which nucleate and elongate non-branched actin filaments, participate in the assembly of structures promoting leading-edge extention of migrating cells. Despite recent advances, little is known relative to how these two classes of actin nucleators collaborate at the leading edge. We have tested the idea that GTPase-activated actin assembly mediated by WASp and mDia proteins is coordinated by factors that either modify their activities or bridge the two proteins within cells. One potential 'bridging' factor was Diaphanous-interacting protein (DIP)- also known as WASp-interacting SH3 protein (WISH). DIP binds to both WASp or the related N-WASp and mDia1-2 proteins. The interaction with mDia2 was mediated by a conserved C-terminal leucine-rich domain that bound to the C-terminus of mDia2 bearing the FH2 domain, the critical region of the formin necessary for actin nucleation. DIP also interacts with the respective proline-rich regions of both mDia proteins and WASp. Through a series of approaches, including fluorescence resonance energy transfer (FRET), mDia2 binds to DIP at the leading edge of motile cells and bridges a complex containing both mDia and WASp proteins. Functional experiments show that mDia, WASp and N-WASp collectively regulate actin-membrane dynamics: live cell confocal microscopy demonstrated that similar to the expression of dominant negative mDia, DIP induced profound non-apoptotic membrane blebbing when co-expressed with Cdc42V12 (an activator of mDia2 and N-WASp) in a panel of adherent and non-adherent cells. Collectively, these data suggest that the direct interaction of DIP with either WASp or mDia2 spatially regulate the actin cytoskeleton in profoundly different manners and point towards a fundamental role for DIP as a regulator of mDia2-mediated membrane remodeling at the leading edge of the cell.

2472

**Exploring Rab-regulated Cytoskeleton Remodeling in Drosophila Development**

J. Zhang, M. Scott; Departments of Developmental Biology, Genetics, and Bioengineering, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA

The F-actin cytoskeleton of most eukaryotic cells is dynamically assembled to provide the force for cell morphology change, cell movement and cytokinesis, which are all critical steps for the organism's normal development, growth, survival, and cellular functions. Rab proteins form the largest subfamily of Ras-like GTPases with more than 70 mammalian members and are previously identified as critical regulators of vesicle and

membrane trafficking. Recent data suggest Rabs may play much broader roles, such as mediating intracellular signal transduction. In this study, we identified a previously uncharacterized Drosophila Rab protein, dRab35, as an essential regulator of cytoskeleton remodeling during Drosophila development. Transgenic flies with dominant negative (DN) but not wild-type (WT) dRab35 introduced into flies caused a striking defect in bristle morphology, probably due to inappropriate actin and/or microtubule organization. Dramatic cell morphology changes were observed when yellow fluorescent protein (YFP)-labeled WT, DN, and CA forms of dRab35 were introduced into Drosophila S2R+ cultured cells. A detailed study of how dRab35 regulates actin remodeling is being conducted.

## Centrosomes II (2473-2491)

2473

### A Novel Role for Beta-Catenin in Centrosome Duplication

S. Bahmanyar, W. J. Nelson, A. I. M. Barth; Molecular and Cellular Physiology, Stanford School of Medicine, Stanford, CA

Centrosomes are comprised of a pair of centrioles that duplicate once per cell cycle to give rise to bipolar spindles required for correct segregation of chromosomes in mitosis. Hyperamplified centrosomes are common to many types of cancers and are thought to give rise to multipolar spindles that contribute to genomic instability. How hyperamplification of centrosomes arises in cancer is not known. Here, we show that wild type β-catenin is an integral component of normal centrosomes in interphase cells and the Armadillo repeat domain mediates β-catenin localization to centrosomes. Depletion of β-catenin inhibits centrosome reduplication in hydroxyurea treated U2OS cells indicating a physiological role for β-catenin at centrosomes. Accumulation of a stabilized form of β-catenin, found in many cancers, induces increased centriole splitting in G1/S phase and formation of extra γ-tubulin structures that are initially defective in microtubule (MT) nucleation. Cells that accumulate stabilized β-catenin with extra γ-tubulin structures progress through the cell cycle and gradually acquire greater than two MT-nucleating centrosomes leading to mitotic cells with multipolar spindles. A transcriptionally inactive form of stabilized β-catenin also gives rise to extra γ-tubulin structures indicating that β-catenin is acting structurally rather than transcriptionally to affect centrosome organization. We suggest that there is a threshold level of β-catenin at centrosomes for normal function. Increasing that level results in prolonged centriole separation in G1/S phase and increased centrosome duplication. Mature extra γ-tubulin structures result in spindle defects that could contribute cell transformation by promoting genomic instability.

2474

### When does DNA Damage-induced Centrosome Amplification Occur in Human Cells?

H. Dodson, L. J. Jeffers, C. Morrison; Department of Biochemistry and NCBES, National University of Ireland - Galway, Galway, Ireland

Aberrant centrosome numbers are commonly observed in cancers and in cells with defective DNA repair. Our recent work revealed that centrosome amplification occurs in response to DNA damage caused by ionising radiation or depletion of the Rad51 recombinase (Dodson *et al*., 2004, EMBO J. 23:3864-73). Initial cell cycle analysis showed that this DNA damage-induced centrosome amplfication occurs without an additional round of DNA replication or failed cytokinesis. Therefore, we hypothesised that the centrosome amplification occurs during a prolonged G2 phase after DNA damage. In order to test this idea and to define the cell cycle stage at which centrosomes duplicate after irradiation, we are examining DNA damage-induced centrosome amplification in live cells. In response to gamma-irradiation, the human U2OS cell line shows increased numbers of centrosomes, as visualised by immunofluorescence microscopy of gamma tubulin in fixed cells. We have generated U2OS clones that stably express GFP-cyclin B1, cyclin E-GFP, and GFP-PCNA, which can serve as temporal markers of the cell cycle in live cells. To visualise centrosomes, we have generated centrin-GFP-expressing clones and are optimising the expression of centrin tagged with monomeric RFP (mRFP). The cell cycle markers may also be tagged with mRFP. Using these tools we propose to follow live cells after ionising radiation and to define centrosome amplification with respect to cell cycle stage using PCNA, cyclin B, cyclin E and possibly cyclin A. These experiments will define the relationship between the chromosome and centrosome cycles under conditions where they become decoupled after DNA damage.

2475

### Cell Cycle Control of Centrosome Duplication

M. Tsou, T. Stearns; Biological Sciences, Stanford University, Stanford, CA

The centrosome duplicates once per cell cycle following a stereotyped series of steps. During G1/S, new centrioles form closely juxtaposed to each of the two original centrioles. The new centrioles remain in a tightly opposed orthogonal configuration during S, G2, and early M phase. These centrioles become disengaged at the end of mitosis or early G1, losing their strict orthogonal configuration, but still joined by an uncharacterized flexible linker. Recent results indicate that there is a centrosome-intrinsic block to reduplication of centrosomes during a single cell cycle. These experiments used cell fusion between combinations of G1, S, and G2 phase cells to show that centrosome duplication only occurs when the fusion products contained previously unduplicated G1 centrosomes. The simplest model to account for this block is that there is a difference between unduplicated G1 centrosomes and duplicated G2 centrosomes such that centrosomes that have already duplicated are not competent to duplicate again within a single cell cycle. To understand this difference, we are using Xenopus egg extract to develop an in vitro system for centrosome duplication that recapitulates this control. By manipulating the duplication potential of centrosomes within the extract, we seek to identify factors that are required for the block to reduplication.

2476

### A Novel Role for TGF-β in Regulating Centrosomal Amplification in Non-malignant Human Mammary Epithelial Cells

C. A. Maxwell, R. Gupta, A. Erickson, M. H. Barcellos-Hoff; Cancer Biology, Lawrence Berkeley National Laboratory, Berkeley, CA

Ionizing radiation (IR) induces genomic instability that may be offset by concomitant TGF-β activation. Centrosome abnormalities (CA) are implicated in the origin of chromosomal instability and are frequently present in cancer cells and premalignant lesions. Here, we investigate whether IR induces CA in non-malignant, p53-competent, human mammary epithelial cells (HMEC) and determine the influence of TGF-β. Following irradiation at graded doses (10-500cGy), either S1 or MCF10A HMEC grown in serum-free conditions were supplemented (+/-TGF-β; 400pg/ml) at 48 hour intervals and CA were quantified one week post-IR. Surprisingly, both HMEC are extremely sensitive to IR-induced CA (IRCA). IRCA occurs at low dose (10cGy), is dose dependent and is significantly inhibited by addition of TGF-β. In cancer cells, IRCA occur

during G2 growth arrest and are thought to precede mitotic catastrophe as another defense against genetic damage. These late time-point analyses suggest that IRCA persist within irradiated populations and do not herald mitotic death. To test this, HMEC were clonally propagated (+/-TGF-β) following IR (200cGy). Clonal propagation alone, as well as irradiation, significantly increased CA in a manner inhibited by TGF-β. To differentiate whether TGF-β PREVENTS or SUPERVISES stress-induced CA, IRCA were examined at early time-points (0-72hr). TGF-β did not prevent IRCA, which occur within one cell cycle independent of treatment and without an associated cycle delay. These data strongly argue that exogenous TGF-β SUPERVISES CA resulting from cellular stress. Consistent with this hypothesis, the frequency of spontaneous CA is significantly higher in *Tgfβ1* null compared to heterozygous keratinocytes and in HMEC treated with TGF-β neutralizing versus control antibodies. In conclusion, p53 competent HMEC are extremely sensitive to CA, HMEC with CA are replication competent and TGF-β is essential for supervising cells containing spontaneous and stress-induced CA.

2477

**Centriole Copy-number Control System**

W. Marshall, J. Feldman; Biochemistry, UCSF, San Francisco, CA

Cells maintain a precise centriole copy number of two per cell. We asked whether this precision might reflect an error-correction mechanism, by examining centriole number in Chlamydomonas mutants with defects in centriole segregation. We found that cells appear to modulate the efficiency of centriole duplication as a function of copy number. Dynamical systems modelling of centriole inheritance indicates that number-dependent duplication coupled with de novo assembly leads to a robust copy number control system. Further modeling studies predict the phenotype to be expected for mutants defective in the specific processes of de novo assembly, templated duplication, or copy-number dependent duplication.

2478

**Centriole Function in Cytokinesis**

E. S. Kannegaard, W. F. Marshall; Biochemistry, University of California San Francisco, San Francisco, CA

Centrioles were the subject of intense research in the early days of cell biology due to their central location within the cell division machinery. However, despite a century of research, the function of centrioles in cell division remains unknown. Previous studies have suggested a direct role for centrioles in cytokinesis by correlating centriole movement with resolution of cytokinesis and an indirect role via the implication of astral microtubules in contractile ring positioning and direction of new membrane deposition. The unicellular green alga *Chlamydomonas reinhardtii* is an ideal system to further study the direct function of centrioles in cytokinesis because it undergoes a closed mitosis and there are a number of strains available that are mutant in centriole number and structure that can be used to probe the precise role of centriole. Additionally, the recently published centriole proteome provides a wealth of candidate proteins that may be tested for functional roles through RNAi knockdown studies. Preliminary evidence suggests that centriole number mutants exhibit increased rates of cytokinetic failure as compared to wild type cells.

2479

**Repeated Duplication of the Centrosome during S-phase Does Not Require Microtubules**

E. S. Halpin, J. Young, E. H. Hinchcliffe; Biological Sciences, Notre Dame, South Bend, IN

Centrosome duplication normally occurs once per cell cycle, as each centriole assembles a daughter or pro-centriole. While centriole number determines the number of centrosomes, it remains unclear whether or not centrosomes can duplicate in the absence of centriole elongation. To test this, we use Chinese hamster ovary (CHO) cells arrested in S-phase, which are capable of undergoing repeated rounds of duplication without coordinate cell cycle progression. We then inhibit microtubule polymerization, allowing the centrosome duplication cycle to continue but preventing the elongation of centrioles. We assay for centrosome number by immuno-labeling with anti-gamma tubulin and by GFP-centrin. We find that during 60 hrs of S-phase arrest in the absence of microtubules, multiple gamma tubulin-containing structures form, often close to the nuclear envelope. These structures do not contain centrioles. When the colcemid is washed out, each of these structures assembles a morphologically normal centrosome that nucleates a microtubule aster. Furthermore, when the colcemid is washed out and the S-phase arrest is released, these structures will go on to form functional mitotic spindle poles. We show that cells treated in this fashion can assemble up to 48 distinct spindle poles. Importantly, the number of “centrosomes” that assemble following colcemid wash-out is similar to the number of true centrioles that assemble during S-phase arrest alone (i.e. with microtubules). Our findings reveal that the re-duplication of centrosome during S-phase arrest does not require an intact microtubule network. Our work also suggests that there is a duplication event that repeatedly occurs during S-phase that does not require the formation or elongation of daughter centrioles. Such a duplication event could represent the formation of a centrosome precursor structure, which has the capacity to nucleate a true centriole-containing centrosome.

2480

**The Duplication of the Centrosome Does Not Require Cell Cycle Progression into S-phase in Mammalian Cells**

T. M. Durcan, T. Rao, E. H. Hinchcliffe; Biological Sciences, University of Notre Dame, South Bend, IN

In animal cells, centrosome duplication occurs once per cell cycle, and is commonly thought to initiate near the G1/S phase transition or during S phase. However, evidence from sea urchins suggests that centrosome duplication can occur prior to the onset of S-phase. We examined the ability of Chinese hamster ovary cells stably transfected with GFP-centrin arrested prior to the onset of S phase to duplicate their centrioles. The GFP-centrin allows for the visualization of centriole number before and after duplication. To arrest cells prior to S phase, cells were initially synchronized in G0 by serum starvation then released in media containing serum and the well-characterized G1 inhibitor mimosine. After 48hrs in G1, 97% of cells had undergone centrosome duplication: 46% possessed 4 centrioles, whilst 51% possessed 5 or more centrioles. G1 arrest was confirmed by flow cytometry, bromodeoxyuridine (BrdU) incorporation, and time-lapse video microscopy. This is in contrast to CHO cells arrested in S phase with aphidicolin - 90% of S-phase cells contained greater than 5 centrioles, with many cells having greater than 14 centrioles. We also repeated these experiments in the non-transformed Chinese Hamster Embryonic Fibroblast (CHEF) cell-line; centriole number was assayed using a centrin antibody. 53% of CHEF cells arrested with mimosine were found to possess 4 centrioles after 48 hrs; we did not find greater than 4 centrioles in CHEF cells. Centriole duplication in G1-arrested cells was inhibited by addition of the Cdk2 inhibitor roscovitine; 67% of CHO cells

contained 2 centrioles after 48hrs in G1 + roscovitine, and only 6% contained more than 4 centrioles. Thus, we conclude that both transformed and un-transformed mammalian cells do not require the onset of S-phase in order to undergo centriole duplication.

2481

**Regulation of Centrosome Duplication by SCF Ubiquitin Ligases**

G. C. Rogers, S. L. Rogers; Biology, University of North Carolina, Chapel Hill, NC

During mitosis in higher eukaryotic cells, centrosomes organize the poles of the mitotic spindle in order to guide segregating chromosomes to each daughter cell. However, in cells that have either too many or too few centrosomes, chromosome segregation may occur asymmetrically (on a multipolar spindle) or may not occur at all (on a monopolar spidle), resulting in aneuploid cell divisions and genomic instability. In order to ensure proper chromosomal inheritance, cells must duplicate the centrosome only once per cell cycle. Although we know that the regulatory pathways that govern centrosome duplication are closely tied to cell cycle progression, the molecular components involved remain largely unknown. Recent genetic analyses of centrosome duplication in *Drosophila* have implicated ubiquitin-mediated protein degradation as mutations in subunits of the SCF E3 ubiquitin ligase (SkpA and the F-box protein, Slimb) result in the production of supernumerary centrosomes . To expand upon these initial findings, we have conducted a functional genomic analysis of SCF components using RNAi in cultured *Drosophila* cells. Using a bioinformatic approach, we find that the *Drosophila* genome encodes 42 F-box proteins, 7 Skp subnits, 6 Cullin subunits, and 3 Roc protein family members. Systematic inhibition of these genes using RNAi has replicated the mutant phenotypes of known genes, with respect to centrosome duplication and cell cycle progression, and has implicated novel genes in these processes, as well. We will present our bioinformatic analysis of the SCF ubiquitin ligase subunits along with our phenotypic analyses using RNAi.

2482

**Aurora-A Activates D-TACC/Msps Complexes Exclusively at Centrosomes to Stabilise Centrosomal Microtubules during Mitosis**

T. Barros,[1] K. Kinoshita,[2] A. Hyman,[3] J. W. Raff[1]; [1]The Gurdon Institute, University of Cambridge, Cambridge, United Kingdom, [2]The Gurdon Institute, Max-Planck-Institute of Molecular and Cell Biology and Genetics, Dresden, Germany, [3]Max-Planck-Institute of Molecular and Cell Biology and Genetics, Dresden, Germany

Centrosomes are the dominant sites of microtubule (MT) assembly during mitosis in animal cells, but it is unclear how this is achieved. The transforming acidic coiled-coil (TACC) proteins stabilise MTs during mitosis by recruiting Msps/XMAP215 proteins to centrosomes. TACC proteins can be phosphorylated *in vitro* by Aurora-A kinases, but the significance of this remains unclear. We show that *Drosophila* TACC (D-TACC) is phosphorylated on Ser863 in an Aurora-A-dependent manner exclusively at centrosomes during mitosis. In embryos expressing only a mutant form of D-TACC that cannot be phosphorylated on Ser863 (GFPS863L), spindle MTs are only partially destabilised, while astral MTs are dramatically destabilised. GFPS863L is concentrated at centrosomes, and recruits Msps there, but cannot associate with the minus ends of MTs. We propose that the centrosomal phosphorylation of D-TACC on Ser863 allows D-TACC/Msps complexes to stabilise the minus ends of centrosome-associated MTs. This stabilisation is essential for the stabilisation of centrosomal MTs, but is not essential for the stabilisation of spindle MTs, presumably because an acentrosomal pathway of spindle assembly can largely compensate for the destabilisation of the centrosomal MTs. Our findings suggest that the phosphorylation of D-TACC by Aurora A ensures that centrosomes are the dominant site of MT assembly during mitosis.

2483

**Distinct γ-tubulin Complexes in Flies: Does Form Follow Function?**

C. Wiese, M. Tremaine, P. Dobrzyn; Dept. of Biochemistry, University of Wisconsin-Madison, Madison, WI

γ-Tubulin is an indispensable component of the animal centrosome. Within the cell, it exists in a multi-protein complex containing between two (yeast) and seven or more (frogs, flies, humans) additional proteins named gamma ring proteins (Grips/GCPs). Most of the components of the metazoan γ-tubulin complex (named γTuRC) are highly conserved among phylogenetically distant species. Curiously, many organisms (including humans and our model system, *Drosophila*) have two separate γ-tubulin genes that are developmentally regulated. In flies, one γ-tubulin isotype is expressed only in the ovary and functions during early embryonic cell divisions. It is subsequently replaced by the other isotype, which persists in somatic tissues throughout the adult fly. While the complexes formed by the embryonic γ-tubulin have received much attention, little is known to date about the γ-tubulin that nucleates and organizes microtubules during most of the life of the organism, or about the proteins that interact with this γ-tubulin. Similarly, little is known about how molecular differences between the two γ-tubulins might influence their function. In this study we isolated γ-tubulins from *Drosophila* embryos or tissue culture cells and discovered that they form discrete complexes with similar but distinct compositions. Surprisingly, each γ-tubulin isotype associates with a different splice variant of one of the Dgrips, Dgrip84/GCP2. This may indicate a role for Dgrip84/GCP2 in regulating the activity and/or the location of the different γ-tubulin complexes.

2484

**Ste20-related Mammalian Protein Kinase Losk is Involved in Development of Cellular Microtubule Radial Array**

A. V. Burakov,[1] O. V. Kovalenko,[1] L. A. Zinovkina,[1] E. S. Potekhina,[1] O. N. Zhapparova,[2] S. A. Kuznetsov,[2] E. S. Nadezhdina[3]; [1]Biochemistry, Beloserskiy Institute of Physico-Chemical Biology, Moscow, Russian Federation, [2]Molecular Biology, Lomonosov Moscow State University, Moscow, Russian Federation, [3]Cell Biology, Institute of Protein Research, Russian Academy of Science, Moscow, Russian Federation

Ste20-related serine-threonine protein kinase LOSK has been identified as a microtubule- and centrosome- associated protein. Bacterialy expressed LOSK N-terminal catalytic domain (LOSK-DT) is active *in vitro* and phosphorylates histone H1 and MBP. $LOSK^{K63R}$-DT is a competitive inhibitor of LOSK-DT activity. Over-expression of LOSK-DT in Vero cells had no obvious effect to microtubule organization, but over-expression of $LOSK^{K63R}$-DT led to a disoranization of radial microtubule array and inhibition of microtubule re-growth from the centrosome after their depolymerization by chilling. Conversely, $LOSK^{K63R}$-DT over-expression did not influence microtubule-polymerizing activity of centrosome *in vitro*. The effect of $LOSK^{K63R}$-DT was prominent also in CHO cells and less clear in HeLa where control microtubules were not organized into radial array. Both LOSK-DT and $LOSK^{K63R}$-DT induced equial rate of apoptotic events, did not influence actin cytoskeleton and caused moderate reduction of gamma-tubulin and pericentrin abundance in centrosome. However, $LOSK^{K63R}$-DT over-expression induced much more significant

decrease of centrosomal dynamitin and active (recognized with monoclonal antibody) form of $p150^{Glued}$ than over-expression of LOSK-DT. Thus, protein kinase LOSK is involved into centrosomal dynactin regulation, and its activity is required for both the development and maintenance of the microtubule radial array in certain cell types.

2485

**Centriole Generation during Ciliogenesis in Airway Epithelial Cells**

E. Vladar,[1] T. Stearns[1,2]; [1]Department of Genetics, Stanford University School of Medicine, Stanford, CA, [2]Biological Sciences, Stanford University, Stanford, CA

Ciliated epithelial cells generate hundreds of centrioles/basal bodies during differentiation. Thus these cells must overcome the normal controls that limit centriole duplication to one round per cell cycle in dividing cells. Ciliated epithelial cells present an ideal model system for understanding both the structural requirements for centriole formation, and the regulatory mechanisms that normally limit centriole number. To study centriole generation in ciliated epithelial cells we set up a primary mouse culture system that allows ciliogenesis to occur *in vitro*. Cells for this culture are derived from trachea of a transgenic mouse strain expressing GFP under the control of a ciliated cell-specific promoter. In these cultures, ciliating cells are GFP-positive even before the appearance of ciliary axonemes, allowing sorting for an enriched population during the stages of ciliogenesis. Immunofluorescence showed that many known structural and functional components of the centrosome also localize to the basal body of the cilia. We have characterized the dynamics of these proteins during ciliogenesis by light and electron microscopy. To investigate the function of these proteins at the basal body, we developed a method for efficient lentiviral gene transfer into cultured ciliated epithelial cells. Lentiviral constructs are being used to validate the localization of centrosomal proteins at the basal body using GFP fusion proteins, and to test the effect of perturbing expression levels for several candidate proteins by overexpression or RNAi-mediated depletion.

2486

**Molecular Analysis of Centriole Assembly in C elegans**

A. Dammermann,[1] I. McLeod,[2] J. R. Yates,[2] K. Oegema[1]; [1]Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA, [2]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

Centrioles recruit pericentriolar material to form centrosomes and template the formation of cilia. Their duplication, which occurs once per cell cycle in dividing cells and during ciliogenesis in differentiated cells, proceeds in a number of discrete steps, which have been extensively characterized at the morphological level. However, very little is known about the molecular basis underlying these events. We have previously used a fixed assay based on incorporation of the centriolar structural protein SAS-4 to examine the molecular requirements for centriole assembly in the C. elegans early embryo. Of the 7 proteins currently known to be required, 5 (SAS-4, -5, -6, ZYG-1 and SPD-2) localize exclusively or primarily to centrioles. To further characterize their role in centriole duplication, we are examining their recruitment to centrioles during the first embryonic division in live embryos. Our results suggest that these proteins act at distinct stages in the duplication process. In addition, we are pursuing biochemical means to characterize the nature of the complexes within which these proteins are found. Mass spectrometry performed on complexes isolated by tandem affinity purification has so far yielded one novel centriolar protein, validating this approach.

2487

**The SUN Domain of Mps3 is Required for Formation of a Function Spindle Pole Body in *Saccharomyces cerevisiae***

S. L. Jaspersen, S. N. Shelton; Stowers Institute, Kansas City, MO

SUN domain-containing proteins (for Sad1 UNC-84 homology) are a family of structurally conserved inner nuclear envelope proteins that share an approximately 150 amino acid region of homology in their C-termini. SUN proteins have been identified in the proteomes of virtually all eukaryotes, and we have recently shown that the budding yeast spindle pole body (SPB) component Mps3 is structurally and functionally related to Sad1, a *Schizosaccharomyces pombe* SPB protein that contains a SUN domain. We identified essential residues within the Mps3 SUN domain through mutational analysis and demonstrated that this domain is important for Mps3 function during SPB duplication, nuclear migration following mating and establishment of sister chromatid cohesion. Genetic and cytological analysis of Mps3 SUN domain mutants indicated that the SUN domain is required for formation of a functional SPB possibly by tethering proteins in the nuclear envelope. Consistent with this hypothesis, we have found that overproduction of another membrane component of the SPB, Mps2, rescues the growth defect of Mps3 SUN domain mutants. We currently are investigating the possibility that Mps2 and Mps3 physically interact through the Mps3 SUN domain and are examining in greater detail the effect that mutation of the Mps3 SUN domain has on SPB structure. The importance of the Mps3 SUN domain for SPB duplication and integrity raises the interesting possibility that vertebrate SUN proteins may be involved in maintaining a physical association between centrosomes and the nuclear envelope.

2488

**Proteomic Characterization of Early Basal Body Assembly in *Tetrahymena thermophila***

C. G. Pearson,[1] C. L. Kilburn,[1] E. P. Romijn,[2] J. R. Yates,[2] M. Winey[1]; [1]MCDB, CU at Boulder, Boulder, CO, [2]Dept of Cell Biology, Scripps Research Institute, San Diego, CA

Centrosomes promote the fidelity of chromosome transmission and aberrations in their number and structure is evident in the majority of tumors. Integral to centrosome duplication and organization, centrioles are a core structure around which centrosomes organize. Basal bodies, for the formation of cilia and flagella, are analogous to centrioles with a conserved molecular composition and structural organization. Initial events in the assembly of nascent centrioles and basal bodies are highlighted by the formation of an amorphous disk and cartwheel followed by the microtubule rich blades to form a barrel structure. Centriole study is constrained by the limited number of known components. To identify the constituents that generate these early steps in assembly, we have utilized a preparation that enables cartwheel reassembly, *in vitro* (Gavin, 1984). Basal body enriched, biochemical preparations were isolated from *Tetrahymena* and placed into cartwheel reassembly conditions. Structures resembling early steps in basal body and centriole assembly were reassembled and subjected to mass spectrometry (MudPIT) to identify basal body components necessary for cartwheel assembly. We are currently localizing these components using GFP fluorescence imaging and high resolution immuno-EM. Furthermore, using a subtractive proteomic approach, proteins dependent upon γ-tubulin for localization to basal bodies were also identified. γ-

tubulin is essential for the maintenance of basal bodies in *Tetrahymena* (Shang et al, 2002), and its depletion was used to generate enriched samples with and without basal bodies. MudPIT with subtractive computational methods were used to identify components that require γ-tubulin for association with basal bodies. In addition to predicting amorphous disk and cartwheel proteins, these studies identified a number of known and potentially novel basal body and centriole components whose analysis will elucidate the assembly pathways for basal bodies and, ultimately, centrioles.

2489

**The *UNI2* Gene in *Chlamydomonas reinhardtii* Encodes a Component of Basal Bodies and Probasal Bodies**

B. P. Piasecki, W. Wu, M. LaVoie, L. W. Tam, P. A. Lefebvre, C. D. Silflow; Plant Biology, University of Minnesota, St. Paul, MN

The unicellular biflagellate alga *Chlamydomonas reinhardtii* provides a model system for the study of eukaryotic basal bodies/centrioles. Among strains with aberrant flagellar number generated by insertional mutagenesis, we identified mutants at the *UNI2* locus with approximately 50% aflagellate cells, 25% uniflagellate cells, and 25% biflagellate cells. The majority of uniflagellate cells fail to assemble the younger of the two flagella, positioned cis to the eyespot. The mutant cells contain ultrastructural defects in the transition zone at the base of the axoneme. The gene containing these mutations was identified using DNA rescue. The gene transcript, deduced from RT-PCR experiments, encodes a predicted protein with a molecular mass of 134 kD. A predicted rat protein shows significant homology with the Uni2p in two regions. Both proteins are predicted to have a similar isoelectric point and to contain a coiled-coil domain. The phenotype of a null *uni2* mutant allele was rescued to wild-type with a gene construct containing an epitope tag. Immunofluorescence microscopy of the tagged protein showed localization at positions coinciding with basal bodies and probasal bodies in $G_1$ cells. Immunogold electron microscopy is underway to identify the subcellular localization of Uni2p. Preliminary results indicate that the protein localizes to the probasal bodies and the transition zones of mature basal bodies. These results suggest that Uni2p assembles on probasal bodies prior to elongation of the triplet microtubules to form the mature basal body. Immunoblot analysis shows Uni2p is present in at least two molecular-weight variants. Treatment of whole-cell extracts with phosphatase converted the protein to a single molecular-weight form, indicating Uni2p is phosphorylated. Preliminary results indicate the phosphorylation pattern of the Uni2p changes during the cell cycle but not during flagellar assembly of $G_1$ cells.

2490

**Septation in the Fission Yeast *Schizosaccharomyces pombe* Requires the Presence of a Single Spindle Pole Body**

V. Magidson,[1] F. Chang,[2] A. Khodjakov[1]; [1]Molecular Medicine, Wadsworth Center, Albany, NY, [2]Microbiology, Columbia University, New York, NY

Cytokinesis in *S. pombe* is regulated by a cascade of GTPase and protein kinases known as the Septation Initiation Network (SIN). This pathway promotes contraction of the medially positioned actin ring and formation of the septum that separates daughter cells from each other. SIN components localize to the spindle pole bodies (SPBs, yeast centrosomes), suggesting that these organelles act as the activation sites for the SIN. To directly test whether SPBs are necessary for cytokinesis, we laser-ablated SPBs at different stages of mitosis. We find that ablation of both SPBs during metaphase (in a mad2 null background) induces disassembly of the actin ring and prevents initiation of cytokinesis. When both SPBs are ablated during early-to-mid anaphase B the ring begins to contract but then disassembles before cytokinesis is completed. Thus, SPB activities are necessary for not only the initiation of ring contraction, but also the maintenance of the actin ring during cytokinesis. It has been shown that some of the SIN components (*e.g.*, cdc7p) associate with only the daughter SPB during anaphase. These observations suggested that only the daughter SPB is responsible for SIN function. To test this hypothesis we ablated just one SPB during metaphase. All of these cells complete cytokinesis normally. Further, when we ablate just the daughter SPB during anaphase B in cells expressing cdc7-GFP, the mother SPB transiently recruits cdc7p and most of these cells complete cytokinesis. Thus, only one SPB (either the daughter or mother) is sufficient for successful cytokinesis. These studies also suggest that the daughter SPB inhibits SIN activation on the mother SPB.

2491

**Mps1 Phosphorylation of the Kinetochore Component Dam1 Alters the Metaphase Kinetochore Arrangement**

D. R. Gestaut,[1] M. M. Shimogawa,[1] B. Graczyk,[1] M. K. Gardner,[2] M. Ess,[1] A. Zelter,[1] C. Ruse,[3] S. Niessen,[3] T. J. Yoder,[1] J. N. Molk,[4] K. Bloom,[4] J. R. Yates,[3] D. J. Odde,[2] T. N. Davis[1]; [1]Department of Biochemistry, University of Washington, Seattle, WA, [2]Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, [3]Department of Cell Biology, Scripps Research Institute, La Jolla, CA, [4]Department of Biology, University of North Carolina, Chapel Hill, NC

Advances in fluorescent protein tagging allow us to observe metaphase in yeast, providing a model system for the study of forces that regulate spindle length and kinetochore positioning. Using computational methods, we can quantitatively compare metaphase kinetochore arrangements and develop models that predict the observed phenotypes. In a screen for mutations that increase the forces on the spindle pole body (SPB), we isolated *DAM1-765*, a dominant allele of *DAM1* that positions kinetochores closer to SPBs at metaphase. Dam1 is a kinetochore protein required for attachment to kinetochore microtubules. The *DAM1-765* allele contains a single serine to phenylalanine substitution at one of several residues phosphorylated by Mps1 kinase. Two of these Mps1 phosphorylation sites play a role in regulating microtubule length. Alanine substitution at either site results in long microtubules and kinetochores adjacent to SPBs. The distributions of tubulin and kinetochores in the mutant can be simulated computationally by decreasing the microtubule catastrophe frequency, which results in long microtubules. We propose that Mps1 phosphorylation of Dam1 is required for proper positioning of metaphase kinetochores.

## Nerve Cell Cytoskeleton (2492-2511)

2492

**Comparative Atomic Force and Optical Microscopy of Neuronal Growth Cones**

E. L. Grzywa,[1] A. Lee,[2] G. U. Lee,[1] D. M. Suter[2]; [1]School of Chemical Engineering and Bindley Bioscience Center, Purdue University, West Lafayette, IN, [2]Department of Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN

The *Aplysia* neuronal growth cone has been established as an excellent model system to study growth cone motility and cytoskeletal dynamics

because of its large size and well defined cytoplasmic regions. While the morphology of these growth cones has been well characterized with optical techniques such as Differential Interference Contrast (DIC) and fluorescence microscopy, there is very little quantitative information on the three dimensional structure of these growth cones available. Therefore, we measured the heights of various growth cone regions using Atomic Force Microscopy (AFM) of fixed *Aplysia* bag cell neurons. The average height of the peripheral domain was determined with 53 nm, while the central domain was in average 1020 nm high. Interestingly, the tips of filopodia were found to be significantly thicker (96 nm) than the base of the filopodia (38 nm). These data correlate well with the visual appearance of growth cone morphology using DIC or fluorescence microscopy. However, a direct comparison of volume determination by AFM and fluorescence intensity measurements of neurons injected with Texas Red-labeled dextran revealed that AFM provides height and volume information with significantly higher resolution and accuracy than fluorescence methods frequently used in cell biology. Our results provide important quantitative information for future analysis of biophysical properties of neuronal growth cones, such as force transduction and protein diffusion, as well of the signal transduction processes underlying neurite outgrowth and guidance.

2493

**Experimental Evidence for Protracted Pausing of Neurofilaments in Axons**

N. Trivedi,[1] P. Jung,[2] A. Brown[1]; [1]Center for Molecular Neurobiology, The Ohio State University, Columbus, OH, [2]Department of Physics and Astronomy, Ohio University, Columbus, OH

Short-term tracking of GFP-tagged neurofilaments in cultured neurons using live-cell fluorescence imaging has shown that the actual rate of movement is fast (average≈0.2-0.3μm/s), whereas long-term tracking *in vivo* using radio-isotopic pulse-labeling has shown that the overall rate is slow (average≈0.003-0.03μm/s). Using computational modeling, we have shown that these data can be explained if we assume that the neurofilaments pause 97% of the time. However, the short-term tracking studies in cultured neurons indicate that the moving neurofilaments pause only 40-70% of the time. One possible explanation for this discrepancy is that our live-cell imaging studies underestimate the true pausing kinetics. Alternatively, neurofilament transport could be faster in cultured neurons than *in vivo*. To test these hypotheses, we investigated neurofilament pausing dynamics in cultured rat sympathetic neurons using a photoactivatable green fluorescent protein fused to rat neurofilament M (PA-GFP-NFM). PA-GFP-NFM protein was activated in a defined axonal region to create a “pulse” of fluorescent neurofilaments, and the “escape” of these neurofilaments from this region was monitored. Time-lapse imaging using this novel “pulse-escape” paradigm revealed the departure of neurofilaments from the activated regions, resulting in a gradual loss of fluorescence. Remarkably, 30% of the neurofilaments remained after 2 hours. The loss of fluorescence was sensitive to metabolic inhibitors, indicating that it was ATP-dependent. Computational simulations revealed that on average the neurofilaments spent 97% of their time pausing, with an average pause duration of 27 minutes. These data show that axonal neurofilaments can pause for very long periods and they demonstrate that short-term tracking of neurofilaments in cultured neurons on a time scale of seconds or minutes greatly underestimates the true pausing behavior. The observed pulse-escape kinetics are consistent with a net transport rate of approximately 0.004μm/s, consistent with slow axonal transport rates *in vivo*.

2494

**An Acto/Myosin Contractile Node Functions to Coordinate Microtubule Dynamics and Entry into the base of Neuronal Growth Cone**

D. T. Burnette, A. W. Schaefer, N. A. Medeiros, P. Forscher; MCDB, Yale University, New Haven, CT

Actin arcs are Myosin II contractile structures in the neuronal growth cone which form in the Transition (T) zone from a condensation of the Peripheral (P) domain’s actin meshwork. We report that the actin arcs are part of a more extensive acto/myosin contractile system that extends proximally into the neurite shaft and coordinates microtubule extension. Fluorescent actin speckle microscopy (FSM) revealed slow (1.5 um/min) but persistent retrograde movement of F-actin structures within the growth cone Central (C) domain that terminated in a Myosin II rich region near the neck of the growth cone. F-actin movement into this region was strongly inhibited by Blebbistatin, suggesting the presence of a Myosin II based contractile node (CN) here. Multimode FSM was used to investigate potential microtubule-actin interactions near the CN. Microtubules entered the growth cone by tracking along actin bundles emanating from the CN. Microtubules in the neurite shaft were more dynamic than CN microtubules. When CN F-actin was depleted with Cytochalasin B, microtubule catastrophe frequency increased. These results suggest the presence of factor(s) in the CN that bias microtubule advance into the growth cone via effects on their assembly dynamics.

2495

**Cytoplasmic Dynein Integrates Microtubules and Actin Filaments during Growth Cone Turning**

I. Tint,[1] Y. He,[2] P. W. Baas,[2] M. M. Black[1]; [1]Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA, [2]Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA

Microtubules extending into filopodia align with bundles of actin filaments during growth cone turning. Here we sought to determine whether dynein-driven forces might play a role in this process. Turning was assayed by culturing rat sympathetic neurons on a patterned substrate consisting of laminin-containing and laminin-free zones. When growth cones reach laminin-free zones, they typically turn to continue elongating. To evaluate the contribution of cytoplasmic dynein, dynein heavy chain was depleted from the neurons using siRNA. Also included in these experiments were neurons depleted of MAP1b, a structural protein that has been implicated in the turning of other types of growth cones. Treatment with siRNA for 2 days reduced MAP1b by ≥95% and dynein heavy chain by ≈70%, after which the neurons were re-plated onto the patterned substrate. Interestingly, depletion of MAP1b had no effect on growth cone turning. In contrast, partial depletion of dynein heavy chain strongly suppressed growth cone turning, such that most growth cones stopped at borders between laminin-containing and laminin-free zones. In dynein-depleted growth cones, microtubules were largely confined to the central zone, often appearing to curve back on themselves, with very few extending into filopodia. By contrast, microtubules in control siRNA-treated growth cones commonly extended into filopodia and aligned with actin bundles. These results suggest that cytoplasmic dynein is instrumental in growth cone turning via on effect on microtubule-actin interactions. Given that cytoplasmic dynein rapidly transports short microtubules down the axon in part by pushing against actin filaments, we speculate that it is essentially the same type of force interaction that accounts for the role of dynein in growth cone turning.

2496

**Microtubule-associated Proteins Regulate the Severing of Microtubules by Katanin and Spastin**

L. Qiang,[1] W. Yu,[1] A. Andreadis,[2] M. Luo,[2] A. Karabay,[1] S. Korulu,[1] P. W. Baas[1]; [1]Drexel University College of Medicine, Philadelphia, PA, [2]Neurobiology Division, Shriver Center, Waltham, MA

The microtubule severing proteins katanin and spastin are important in the neuron for breaking long immobile microtubules into short mobile pieces. It is essential that the severing of microtubules by these enzymes is spatially and temporally regulated so that the entire microtubule array is not severed continuously into subunits. We have hypothesized that fibrous microtubule-associated proteins (MAPs) may regulate the severing of microtubules by restricting the access of katanin and/or spastin to the microtubule lattice. In this view, the severing of microtubules is controlled locally in the neuron by signaling cascades that affect the binding of relevant MAPs to the microtubule. Our goal in the present study was to test whether particular MAPs have the capacity to function in this manner. When katanin or spastin is overexpressed in fibroblasts, the microtubules break into short pieces accompanied by marked depolymerization. However, when the severing proteins are overexpressed together with MAP2c, tau, or MAP4, this effect is markedly attenuated. These three MAPs share a similar microtubule-binding domain. In experiments on tau, the microtubules are partially protected against severing when the microtubule-binding domain alone is expressed, but not when the rest of the molecule absent the binding domain is expressed. The protection is more pronounced with the 4-repeat isoform of tau than the 3-repeat isoform. The protection is not observed when microtubules are stabilized and bundled by taxol, indicating that the presence of the MAP is crucial for the protection. No protection is observed with MAP1b. We conclude that certain MAPs protect microtubules from being severed, while others do not. We are currently using siRNA to deplete MAPs from cultured neurons in order to determine if the microtubules become less resistant to severing proteins in the absence of the MAP.

2497

**Stathmin Family Phosphoproteins and the Microtubule Cytoskeleton in Differentiating Neurons**

F. Poulain,[1,2] S. Chauvin,[1,2] S. Lachkar,[1,2] S. Ozon,[1,2] A. Sobel[1,2]; [1]U706, INSERM, Paris, France, [2]UPMC, Paris, France

Microtubules are major components of the cytoskeleton that regulate essential processes of cell life, including during the different stages of neuronal differentiation. They are particularly dynamic in the growth cone and thus play an essential role in the control of neurite formation. Molecules regulating microtubule assembly and dynamics are thus also key regulators of neuronal differentiation, from early neuritogenesis to process elongation, branching, and structural stabilization. Among such proteins, phosphoproteins of the stathmin family (including SCG10, SCLIP and RB3/RB3'/RB3") share the common ability to sequester tubulin and hence contribute to the control of microtubule assembly and dynamics. Their developmentally regulated expression and their specific localization at membrane compartments of elongating processes and growth cones suggest that they may participate in regulating neuronal differentiation by locally controlling microtubule assembly. In a study aimed at better characterizing the common and specific functional properties of the various proteins of the stathmin family, we found that they are strongly expressed during the first stages of neuron differentiation and specifically targeted to the regions where microtubules are highly dynamic. They are accumulated in the central domain of the growth cone where they appear to be associated with bundled microtubules, and are also observed in the peripheral actin-rich region, in proximity of individual tyrosinated "exploratory" microtubules. Furthermore, pharmacological treatments affecting microtubule organization induced a complete redistribution of the proteins, confirming their close association with microtubule integrity and dynamics in the various growth cone domains. Finally, preliminary functional perturbation experiments, including RNA interference, advocate for a role in neurite outgrowth and differentiation. Altogether, our results suggest that the proteins of the stathmin family are likely regulators of microtubule dynamics within the growth cone and hence participate to the control of process formation during neuronal differentiation.

2498

**Septin Function in C elegans Axonal Migration**

F. P. Finger, C. A. Morton; Biology Department, Rensselaer Polytechnic Institute, Troy, NY

Septins are a family of GTP binding proteins involved in cytokinesis in diverse organisms. They are also expressed in post-mitotic cells, suggestive of other cellular functions. *C. elegans* have two septins, encoded by the *unc-59* and *unc-61* genes. We have recently described a novel role for the worm septins in ventral cord motor neuron axonal migration. To further define the role of septins in axonal migration, we are studying process outgrowth in primary cultures derived from wild-type and septin mutant worm embryos expressing the pan-neuronal *unc-119::GFP* reporter. Extension of processes in *unc-119::GFP* expressing cells derived from wild type embryos begins soon after plating, with approximately 40% of neurons displaying processes after one day in culture. Processes continue to extend and elaborate over the first week in culture, at the end of which approximately 70% of wild type *unc-119::GFP* expressing cells display processes. In contrast, fewer than 20% of *unc-119::GFP* expressing cells derived from null mutant *unc-61(e228)* embryos or from *unc-59(n391)* embryos extend processes during the week in culture. Those processes that are seen in the *unc-61(e228)* cells and in *unc-59 (n391)* cells are shorter and less elaborate than those cultured from wild-type strains. Similar effects are seen when *unc-59* or *unc-61* dsRNA are added to the culture medium. During the first week in culture approximately 70% of cells cultured from the wild-type or septin mutant strains express *unc-119::GFP*, and essentially all cells are viable as revealed by Trypan Blue exclusion. These results are consistent with our previous *in vivo* findings. The actin and microtubule cytoskeletons both display abnormal morphology in septin mutant cultured neurons, suggesting that the septins may regulate cytoskeletal assembly. We hypothesize that the septins are required for establishment and/or maintenance of cortical domains that are necessary for process outgrowth.

2499

**Neurofilaments are Transported Normally along Axons of Cultured Neurons Lacking Myosin Va**

N. Alami, A. Brown; Center for Molecular Neurobiology, The Ohio State University, Columbus, OH

Neurofilaments are transported along axons at slow rates, characterized by fast bidirectional movements interrupted by prolonged pauses. The motors are thought to be dynein and kinesin, which move along microtubules. Recently the Nixon lab reported that neurofilaments also interact with myosin Va (myoVa), which moves along microfilaments, and that neurofilament number and density is increased in axons of dilute lethal *(dl)* mice, which lack myoVa. These data suggest a role for myoVa in neurofilament transport. For example, myoVa could act to increase the frequency of neurofilament movement, perhaps by delivering these polymers to microtubules or by facilitating their association with microtubules in some

other way. According to this hypothesis, the increased neurofilament content of axons in the absence of myoVa could be due to a decrease in neurofilament transport rate. To test this hypothesis, we cultured neurons from superior cervical ganglia of *dl* mice. The neurons extended axons containing neurofilaments and were morphologically indistinguishable from wild type *(wt)*cells. To analyze the transport behavior, we transfected neurons with GFP-tagged neurofilament proteins and tracked neurofilament movement by time-lapse imaging of naturally occurring gaps in the axonal neurofilament array. Statistical comparison of the frequency, directionality, peak velocity, and average velocity including and excluding pauses for 53 neurofilaments in *dl* neurons and 58 neurofilaments in *wt* neurons revealed no significant differences. Thus myoVa has no apparent influence on neurofilament transport in these axons. If myoVa is responsible for short-range longitudinal or lateral movement of axonal neurofilaments in these axons, then any effects of these movements on the transport kinetics are too subtle to detect. We speculate that myoVa may have a more significant effect on neurofilament transport behavior in larger axons with more abundant neurofilaments, such as those encountered *in vivo*.

2500

**Netrin Signaling in Nerve Growth Cones Requires Soluble Adenylyl Cyclase**

K. Y. Wu, J. H. Zippin, M. Kamenetsky, U. Hengst, J. Buck, L. R. Levin, S. R. Jaffrey; Pharmacology, Weill Medical College of Cornell University, New York, NY

The response of growing axons to guidance cues is controlled by cyclic nucleotides. Manipulation of cyclic AMP (cAMP) or its effectors can reverse the polarity of turning responses to molecules such as netrin-1, a secreted molecule that has key roles in the development of the central nervous system. The specific pathways by which cAMP levels are regulated in growth cones and in netrin-1 signaling are unclear. Here we show that soluble adenylyl cyclase (sAC), a noncanonical cAMP-generating enzyme that has previously been found to control sperm facilitation, is expressed in neurons and in developing axons and growth cones. sAC expression in neurons results in an increase filopodial formation and size of growth cones. Furthermore, inhibition of sAC blocks netrin-induced stimulation of growth cone elaboration and chemotropic turning responses of axons to gradients of netrin-1. We also find that netrin-induced cAMP levels increases are mediated by the activation of sAC. Taken together, these results identity a new signaling mechanism for netrin-1, in which sAC is activated and mediates netrin-1 function, and indicate a fundamental role for sAC in axon guidance.

2501

**Frazzled Signaling at the *Drosophila* Embryonic CNS Midline**

J. N. Talbot, M. Sabramanian, M. F. A. VanBerkum; Biological Sciences, Wayne State University, Detroit, MI

Frazzled (Fra) is a key receptor in axon guidance. At the *Drosophila* embryonic midline Fra functions as a chemoattractive receptor guiding exploring neurons across developing commissures. It is likely to regulate actin and myosin dynamics and movement using a variety of signaling pathways. By reducing or over-expressing Fra in a subset of CNS neurons (pCC/MP2 pathway) we provide genetic evidence that several signaling pathways are involved in Fra regulation of myosin. Over-expression of Fra enhances crossovers seen with constitutively active myosin light chain kinase (ctMLCK), while heterozygous *fra* mutations suppress ctMLCK-mediated crossovers. Interestingly, heterozygote mutations affecting Abelson tyrosine kinase (Abl) suppress the frequency of these crossovers. Three Rho family GTPases, Rho, Rac and Cdc42, show ectopic crossovers when co-expressed with Fra; *abl* mutations alter these interactions. Rac and Cdc42 may regulate MLCK or myosin II through p-21 activated kinase (Pak) or indirectly via regulation of actin dynamics. Rho, via its major effector Rho kinase (Rok), alters myosin II activity by inhibiting myosin phosphatase via phosphorylation of its myosin binding subunit. Indeed, we hypothesize that Fra regulates myosin dynamics at the midline through a Rho-Rok dependent pathway and actin dynamics through a Cdc42-Rac dependent pathway. We are testing this hypothesis using profilin and myosin loss-of-function alleles to tease apart the respective roles of actin versus myosin regulation downstream of Fra. Our work in deciphering how activation of Frazzled translates into forward movement of the growth cone at the CNS midline will help our understanding of the mechanisms of cell motility in general.

2502

**Role of Cofilin Phosphocycle by LIM-Kinase and Slingshot in NGF-Induced Neurite Outgrowth**

M. Endo, K. Ohashi, K. Mizuno; Graduate School of Life Sciences, Tohoku University, Sendai, Japan

Neuronal growth cone motility and neurite extension are based on actin filament dynamics. Cofilin plays an essential role for the rapid turnover of actin filaments by severing and depolymerizing actin filaments. Cofilin is inactivated by phosphorylation of Ser-3 by LIM-kinases (LIMKs) and reactivated by dephosphorylation by Slingshots (SSHs). We have previously shown that the overexpression of LIMK1 inhibits growth cone motility and neurite extension, and the overexpression of SSH1L accelerates growth cone motility and neurite extension in nerve growth factor (NGF)-induced neurite outgrowth of dorsal root ganglion (DRG) neurons. Here we report that the regulation of cofilin activity by LIMKs and SSHs is involved in signaling pathways that regulate NGF-induced neurite outgrowth. When undifferentiated PC12 cells were stimulated with NGF, cofilin dephosphorylation and activation of LIMKs were induced at the same time, and the dephosphorylation of cofilin was dependent on SSH1L and SSH2L. RNA interference revealed that LIMK1, LIMK2, SSH1L and SSH2L were required for NGF-induced neurite outgrowth. Furthermore, during NGF-induced neurite outgrowth of DRG neurons, inhibition of LIMKs activity by a synthetic peptide containing a cofilin phosphorylation site suppressed growth cone motility and neurite extension rate, although the growth cones showed well expanding morphology. Immunostaining analysis showed that cofilin concentrated at the leading edge of the control growth cones, and this distribution was enhanced by overexpression of SSH1L. On the contrary, cofilin was diffusively distributed in the growth cones treated with S3-peptide. These findings suggest that the acceleration of cofilin phosphocycle by LIMK and SSH is important in NGF-induced growth cone motility and neurite extension, and the phosphocycle may play a role for the establishment of the polarity of the growth cone.

2503

**Agrin-induced Formation of Filopodia Depends on GAG Chains and Cdc42**

L. Lin, S. McCroskery, J. M. Ross, M. P. Daniels; Cell Biology, NHLBI, NIH, Bethesda, MD

Agrin is a proteoglycan with heparan sulfate (HS) and chondroitin sulfate (CS) glycosaminoglycan (GAG) chains. Transmembrane (TM) agrin is

expressed in the central nervous system, but its functions are poorly understood. Overexpression of TM-agrin in myotubes and hippocampal neurons causes extensive formation of filopodia, while agrin knockdown reduces neuronal filopodia number. (J. Neurosci. 21:9678; ASCB Abstr. 2004, 281). To investigate the role of agrin GAG chains in the induction of filopodia, we created full-length agrin-GFP mutants lacking HS, CS or HS and CS GAG chains by site-directed mutagenesis (to nullify GAG chain attachment sites). Western blots of mutant agrin-GFPs showed narrowing and reduced molecular weight of the ~300-600 kD band representing glycosylated forms. Filopodia formation in transfected COS7 cells was decreased by ~ 70% with the GAG chain mutants compared to wild-type agrin-GFP. In 3-day-old cultures of rat hippocampal neurons transfected with the mutants, the number of filopodia per µm neurite length was decreased 50-60% compared to wild type. We also found that the mean length of the longest neurite was reduced by ~ 50% in neurons transfected with the HS GAG chain mutant. In contrast, the mean length of the longest neurite increased more than 2-fold in neurons transfected with the CS GAG-chain mutant compared to wild type. Furthermore, the number of large growth cones per neuron was reduced ~ 60%. We examined the role of Cdc42 activation in agrin-induced filopodia induction by cotransfecting COS7 cells with agrin and a dominant negative mutant of Cdc42. The dominant negative reduced filopodia formation by 68%. Moreover, activation of wild-type Cdc42 increased strongly in cells co-transfected with TM-agrin. We propose that GAG chain-dependent interactions of agrin with other proteins leads to activation of Cdc42 and resultant formation of filopodia.

2504

### Regulation of Growth Cone Motility and Axon Growth by Akt and Rac

Y. Chen, F. Zhou, W. D. Snider; Neuroscience Center, University of North Carolina, Chapel Hill, Chapel Hill, NC

PI3K signaling is known to be required for NGF-induced axon growth. We have recently described a PI3K-mediated pathway that regulates axonal microtubule assembly via GSK-3β in dorsal root ganglion (DRG) neurons. Surprisingly, the role of PI3K in regulating the actin cytoskeletion in neurons remains largely unknown. In our previous studies (Zhou et al., *Neuron*, 2004), we have shown that a major downstream mediator of PI3K, active Akt, co-localizes with actin filaments in the neuronal growth cone. This result suggests that Akt may play an important role in regulating the actin cytoskeleton in the growth cone. By using immunofluorescence and time-lapse imaging, we show here that specific inhibition of Akt activity in the growth cone of mouse DRG neurons induces dramatic changes in actin organization and growth cone motility. However, these changes in actin cytoskeleton that result from Akt inhibition have no effect on NGF-induced axon growth. We also demonstrate that active Akt co-localizes with the small GTPase Rac, an important regulator of actin dynamics. Over-expression of wild-type Rac in DRG neurons in the presence of NGF leads to dramatic increases in growth cone size. Surprisingly this effect impedes axon growth, suggesting that Rac-induced actin reorganization negatively regulates axon growth. Taken together, our data suggest that Akt and Rac may act in the same pathway to regulate actin dynamics and growth cone motility. However, Akt/Rac mediated actin regulation may play a negative role during axon extension.

2505

### Ral GTPases: New Regulators of Integrin-Dependent Neurite Branching

G. Lalli, A. Hall; MRC Laboratory for Molecular Cell Biology, University College London, London, United Kingdom

Recent studies have uncovered a role for Ral, a Ras-like GTPase, in a variety of cellular processes, such as regulation of cell morphology, vesicle sorting and neurosecretion. In addition, the identification of the exocyst complex as a downstream effector of active Ral indicates that this GTPase could act as a regulator of polarized membrane delivery. The two Ral isoforms, RalA and RalB, display an abundant but non-overlapping punctate pattern in superior cervical ganglia (SCG) neurons, suggesting an association with vesicular compartments. Based on this evidence, we asked if Ral could influence neurite growth, a key neuronal process requiring a precise coordination between the cytoskeleton and the secretory pathway. We transfected or microinjected freshly plated cortical and SCG neurons with constitutively active or dominant negative versions of Ral, or with membrane-targeted Ral guanine nucleotide exchange factors (GEFs). Observation of living and fixed cells shows that active Ral causes a significant increase in neurite branching. In addition, cortical neurons depleted of endogenous Ral by RNA interference (RNAi) are characterized by a reduced branching complexity, further supporting a role for Ral in promoting branching. In our search for extracellular signals leading to Ral activation, we have found, using both Ral-GTP pull-down experiments and quantitative analysis of branching in SCG neurons plated on different substrates, that Ral is necessary for integrin-mediated neurite branching. RalA and RalB promote branching through distinct pathways involving the exocyst complex and phospholipase D (PLD), respectively. Our findings establish a novel role for Ral in the regulation of neurite morphology and provide a cellular context for identifying the downstream effectors of integrin-mediated neurite branching.

2506

### IQGAP3, a Novel Effector of Rac1 and Cdc42, Regulates Neurite Outgrowth through Actin Cytoskeletal Reorganization

S. Wang,[1] T. Watanabe,[1] J. Noritake,[1] K. Sato,[1] M. Fukata,[2] K. Kaibuchi[1]; [1]Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, Nagoya, Japan, [2]Laboratory of Genomics and Proteomics, National Institute for Longevity Sciences, Obu, Japan

Rac1 and Cdc42, members of Rho family GTPases, play critical roles in diverse cellular processes such as axon guidance and neuronal morphogenesis. They exert the physiological functions through the reorganization of cytoskeleton and adhesion molecules in the concert with their effectors. Here, we identified IQGAP3 as a novel effector of Rac1 and Cdc42. We found that IQGAP3 directly binds to actin filaments through its calponin homology domain and is predominantly expressed in brain. In cultured hippocampal neurons, all IQGAP isoforms were detected at the different levels. Each IQGAP showed the specific localization within the growth cones. We also found that the depletion of IQGAP3 inhibits neurite outgrowth and axonogenesis. Furthermore, IQGAP3 was indispensable for the Rac1/Cdc42-induced neurite elongation and branching in PC12 cells. These results indicate that IQGAP3 specifically regulate the neuronal morphogenesis downstream of Rac1 and Cdc42 and each IQGAP has the different physiological implication.

2507

### Modulation of Axonal Protein Synthesis by Local Stimuli

G. G. Williams,[1] D. E. Willis,[1] M. Kendall,[1] J. Zheng,[1] J. L. Twiss[1,2]; [1]Nemours Biomedical Research, Alfred I duPont Hosp for Children, Wilmington, DE, [2]Biological Sciences, University of Delaware, Newark, DE

It has become clear that vertebrate axons are capable of synthesizing proteins and that this localized translation plays a role in axonal growth. In

developing neurons, growth cone turning in response to axonal pathfinding stimuli, including trophic factors and chemorepulsive signals, requires localized axonal protein synthesis (Piper and Holt, 2004). Work from our lab indicates that axonal protein synthesis is activated during regeneration (Zheng et al., 2001; Hanz et al., 2003). We have recently shown that local application of neurotrophins to regenerating sensory axons alters the transport of mRNAs from cell body into axons (Willis et al., 2005). Here we have asked if the translational capacity of regenerating adult rat sensory axons is locally modulated by extracellular stimuli. Nerve growth factor (NGF) increases neurite outgrowth in adult DRG cultures, and growing axons will turn toward a localized source of NGF. Application of lysophosphatidic acid (LPA) to developing neurons causes neurite retraction. Localized sources of NGF and LPA, immobilized onto polystyrene beads, were used to limit stimulation to axonal subdomains of cultured adult rat sensory neurons. Activity of these immobilized ligands was demonstrated by the turning of growing axons toward or away from the NGF- and LPA-coated beads, respectively. Compared to control cultures, LPA induced an increase in phospho-eIF2a immunoreactivity in the axons while NGF caused a decrease in axonal phosopho-eIF2a immunoreactivity. Phosphorylation of eIF2a on serine 51 is typically associated with an overall decrease in cap-dependent translation. Consistent with this notion, LPA and NGF directly modified the local translation of mRNAs in these axons. Thus, axonal protein synthesis is regulated both by modulation of axonal mRNA levels through altered transport and local changes in specific mRNA translation.

2508

**Profilin:actin Operates in Signaling Synapses in the Lamprey Spinal Chord**

S. Grenklo,[1,2] N. Tomilin,[2] E. Evergren,[2] L. Brodin,[2] R. Karlsson,[1] O. Shupliakov[2]; [1]Department of Cell Biology, Wenner-Gren Institute, Stockholm University, Stockholm, Sweden, [2]Department of Neuroscience, Center of Excellence in Developmental Biology, Karolinska Institutet, Stockholm, Sweden

The role of actin dynamics in synaptic signaling was studied by microinjecting a cross-linked variant of profilin:actin into the lamprey giant reticulospinal synapse. This non-dissociable profilin:actin (PxA) blocks actin polymer-forming complexes operating at filament (+)-ends and hence blocks filament growth. After microinjection and subsequent stimulation of the synapses at 5 Hz for 30 min, a significant number of omega-shaped structures were observed at active zones as compared to control synapses. Most of these structures had a size corresponding to synaptic vesicles, although larger profiles were also observed, possibly reflecting actin-dependent dynamic stages in the presynaptic membrane-vesicle interplay captured by the introduced PxA. Isolation and subsequent characterization of neuronal lamprey profilin:actin, demonstrated that it has similar *in vitro* properties in an actin polymerization assay as mammalian profilin:actin, and antibodies generated against the lamprey profilin partly co-distributed with the synaptic marker SV2 in the lamprey spinal cord. Together these observations indicate a role for profilin:actin in the active zone of the nerve synapse, possibly in connection with synaptic vesicle trafficking.

2509

**Disruption of Actin Polymerization Suggests Two Mechanisms of Dendritic Branch Formation in Neurons**

B. G. Changstrom, E. M. Gibbs, G. S. Withers; Biology, Whitman College, Walla Walla, WA

The actin cytoskeleton regulates the complexity of the dendritic field of neurons, but what local cytoskeletal events initiate the formation of a new branch are not known. Here, we tested the hypothesis that changes in actin polymerization are necessary for dendritic branches to form in cultured hippocampal neurons. During early stages of dendritic development (i.e. the first 5 days in culture) an increase in actin-based lamellipodia appears to parallel the addition of higher order branches, with both increasing about 2-fold. To determine if lamellipodial frequency relates to branching, we treated neurons with drugs that disrupt actin polymerization (cytochalasin D, jasplakinolide, or latrunculin-A) as branches formed. Lamellipodia were clearly disrupted within 1 hour, and were not present after 24 hours. After 24-48 hours, higher order branching was reduced by about 2-fold. Dendritic length, however, increased with exposure to cytochalasin or latrunculin. These data suggest that actin dynamics are important for the formation of new branches, but not for net extension. In addition, actin disruption clearly altered branch angle. For example, after 24 hours of drug treatment, over 40% of the dendritic branches occurred at angles less than 40°; 10% of the branches in control cells fell within this range. From these observations, we propose two mechanisms of dendritic branch formation: the first is actin-based and branches arise from lamellipodia on the dendritic shaft; the second is not dependent on actin dynamics or lamellipodia, but perhaps branches arise via a microtubule-based mechanism from the tip of a dendrite. These data also suggest that actin is important in establishing the geometry of the dendritic arbor. Support: NSF CAREER and REU awards (GW)

2510

**Localization of Protein Kinase A to Actin-rich Cytoskeletal Elements in Neuronal Growth Cones**

M. D. Birger, K. J. Gaston, A. K. Howe; Pharmacology, University of Vermont, Burlington, VT

Proper formation of the neural network is dependent upon the specific regulation of growth cone motility and guidance in response to various environmental cues. This guidance is driven by regulation of actin cytoskeletal dynamics and the signal transduction pathways utilized by guidance cues to effect cytoskeletal regulation are under intense investigation. Recent results from our laboratory have shown that the cAMP-dependent protein kinase (PKA) is enriched in the protrusive structures formed at the leading edge of migrating fibroblasts and that this localization is important for chemotactic cell movement. Because it is well established that growth cone guidance can be modulated by PKA signaling, we have examined the sub-cellular location of PKA in growth cones and peripheral edge structures of migrating and differentiating neuronal cells. Initial efforts focused on PKA type II regulatory subunits (RIIα), as these are known to be spatially regulated through interactions with A-kinase anchoring proteins (AKAPs). Immunofluorescence of endogenous RIIα in differentiating N1E-115 and NG108-15 cells revealed that RIIα co-localizes with cortical actin filaments and microspikes in growth cones and peripheral membrane regions. Co-localization with microspikes was also seen in NG108-15 cells expressing an RIIα-GFP fusion protein. Furthermore, point mutation or deletion of the AKAP-binding region of RIIα altered the its localization. These data support the hypothesis that PKA is recruited to cytoskeletal structures in the periphery of neuronal cells where its activity is likely needed to modulate actin dynamics in response to guidance cues.

2511

### Actin Rods Disrupt APP Transport Leading to Localized Aβ Accumulation in Cultured Neurons

M. T. Maloney,[1] J. N. Fass,[1] L. S. Minamide,[1] A. W. Kinley,[1] J. R. Bamburg[1,2]; [1]Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, [2]Program in Molecular, Cellular and Integrative Neuroscience, Colorado State University, Fort Collins, CO

Cargo transport along the cytoskeleton is particularly vulnerable to blockage in axonal and dendritic neuronal processes. Transport defects are a common feature of neurodegenerative diseases including Huntington's Chorea and Alzheimer's. Late stage Alzheimer's disease brain is characterized by extra-cellular amyloid plaques resulting from deposition of secreted amyloid-beta (Aβ), a proteolytic product of amyloid precursor protein (APP). Beta-cleavage of APP occurs in endosomes, which undergo retrograde transport in neurites before releasing their contents extra-cellularly. Transport defects are one of the earliest functional deficits observed in brains of TG2576 mice, a model for Aβ-induced neurodegeneration. Here we show as little as 10nM soluble Aβ oligomers induce the formation of rod shaped aggregates containing ADF/cofilin and actin in neurites of cultured embryonic rat hippocampal neurons. Rods disrupt microtubules and develop vesicular accumulations containing APP, β-secretase, presenilin-1 and β-cleaved APP (the immediate precursor to Aβ) at their ends. We hypothesize that rods disrupt transport and represent a site for localized Aβ production leading to rod induction in surrounding neurites in a progressively degenerative cycle. To test this hypothesis transport is followed in living neurons by adenoviral-mediated expression of APP conjugated to yellow fluorescent protein (APP-YFP). Spontaneous rods are induced and visualized using adenoviral-mediated expression of *Xenopus* ADF/cofilin conjugated to cyan or red fluorescent proteins (XAC-CFP/RFP). Co-expression of APP-YFP with XAC-CFP/RFP demonstrates impaired APP-YFP transport at rods. Future experiments will measure the levels of Aβ produced at rods in two ways: via ratio imaging of immunostained Aβ to total APP in fixed neurons, and by measuring total Aβ content in medium of neurons with and without rods by immuno-assay. (Supported by grants NS40371, NS 43115 and GM35126 from NIH, IIRG-01-2730 from the Alzheimer's Association and a grant-in-aid from Sigma Xi).

## Intermediate Filaments II (2512-2531)

2512

### Intermediate Filament Assembly Probed with Small-Angle X-ray Scattering: Three-Dimensional Structure of Assembly Intermediates

A. V. Sokolova,[1] L. Kreplak,[2] D. I. Svergun,[3] T. Wedig,[4] H. Herrmann,[4] U. Aebi,[2] S. V. Strelkov[2]; [1]Institute of Bioorganic Chemistry, Moscow, Russian Federation, [2]M.E. Mueller Institute, Biozentrum, Basel, Switzerland, [3]EMBL, Hamburg, Germany, [4]German Cancer Research Centre, Heidelberg, Germany

10-nm wide intermediate filaments (IFs) are the least characterised of the three major cytoskeletal filament systems. While crystallographic data on the dimer representing the elementary IF 'building block' have recently become available, little structural detail is known on both the mature IF architecture and their assembly pathway. We have applied solution small-angle X-ray scattering (SAXS) to investigate the *in vitro* assembly of the wild-type human IF protein vimentin and its two assembly-deficient mutants K139C and NΔ30 by systematically varying pH and ionic strength conditions, and complemented these experiments by electron microscopy observations. We show that tetramers, octamers and 32-mers corresponding to the so-called unit-length filaments represent the principal steps along the vimentin assembly pathway. Based on the SAXS data supplemented by the atomic structure of the elementary dimer and additional structural constraints, first ever three-dimensional models for these assembly intermediates are constructed and refined. Together with most recent crystal structures of vimentin fragments, these results represent a major advance towards unveiling the structural basis of both the normal IF function and the mechanism of human disease caused by mutations in IF proteins.

2513

### Cryo-Electron Tomography of *In Vitro* Assembled Vimentin Filaments

K. N. Goldie,[1,2] A. Hoenger,[1] H. Herrmann[3]; [1]Structural and Computational Biology, EMBL, Heidelberg, Germany, [2]School of Biological Sciences, The University of Auckland, Auckland, New Zealand, [3]Division of Molecular Genetics, German Cancer Research Center, Heidelberg, Germany

Of the three major cytoskeletal systems found in eukaryotic cells, which includes microtubules (MTs) and actin microfilaments (MFs), the structural architecture of intermediate filaments (IFs) remains the least understood. Structurally, IFs are much more diverse than MTs and MFs. Recently employed methods such as chemical cross-linking, X-ray fibre diffraction, site-directed spin labeling and electron paramagnetic resonance have given us new insights into the successive assembly steps of the IF molecule up to the tetrameric level. X-ray crystallographic studies on short fragments are also expanding our structural knowledge, but the arrangement of higher order oligomers within the full-length filament is still unknown. With the aim to resolve filament architecture for a typical IF at the molecular level, we have employed cryo-electron microscopy (cryo-EM) and cryo-electron tomography (CET) to *in vitro* assembled recombinant human vimentin. Typically, vimentin is expressed in mesenchymal cells of higher vertebrates and it is able to form homo-polymeric filaments in the test tube without the need for any cofactors. Cryo-EM allows observation of complex biological systems at close to physiological conditions. We demonstrate that unfixed cryo-EM preparations exhibit a mixed population of compact and loose filament morphologies, which clearly contrasts the findings of most EM studies on conventionally fixed, negatively stained filaments. The filaments exhibit a right-handed twist and in some cases, where they show partial unravelling, four distinct protofibrils can be disseminated. This is in agreement with the four protofibril, (each composed of four dimers) model [Herrmann H. and Aebi U. (2004) Annu. Rev. Biochem., 73:749-89]. Thus, our results provide the first EM-derived structural information in 3-D of *in vitro* assembled vimentin filaments under near physiological conditions and contribute to an increased understanding of the IF architecture at molecular resolution.

2515

### PKCζ is Required for the Shear Stress-Induced Structural Reorganization of Keratin Intermediate Filaments in Alveolar Epithelial Cells (AEC)

S. Sivaramakrishnan, N. Na, R. G. Goldman, K. M. Ridge; Northwestern University, Chicago, IL

Keratin intermediate filaments (KIF) in AEC undergo structural reorganization when subjected to shear stress. Since the assembly state of KIF is

known to be influenced by phosphorylation we determined whether known phosphorylation sites are involved in their reorganization. Specifically, the role of K18pSer33 in the structural reorganization of the KIF network in human AEC was studied in response to shear stress. Shear stress (30 d/cm$^2$, 1h) induced the reorganization of the KIF network from thin cytoplasmic fibers to thick wavy bundled filaments or tonofibrils as determined by immunofluorescence. Ratio imaging of K18pSer 33 to K18 immunofluorescence revealed that the increase in phosphorylation was associated primarily with the tonofobrils observed under shear stress. These results were confirmed by keratin-enriched cytoskeletal preparations from control and shear stress AEC. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with anti-K18 and anti-K18pSer33. There was a three-fold increase in K18pSer33 in shear stressed AEC. The phosphorylation of K18pSer33 under shear stress was inhibited by pre-treatment with 10μM bisindolymaleide (protein kinase C [PKC] inhibitor) but not by U0126 (Erk 1/2 specific inhibitor). Inhibition of classical and novel PKC isozymes by a phorbol ester (PMA, 1μM, 24hrs) did not prevent K18pSer33 phosphorylation under shear stress. Shear stressed AEC expressing a dominant-negative mutant of PKCζ (AEC DN PKCζ) showed reduced K18pSer33 phosphorylation. The AEC DN PKCζ did not show the structural reorganization of the KIF network under shear stress, rather, the KIF network remained as thin fibers. These results show that PKCζ mediated K18pSer33 phosphorylation is required for the shear stress induced structural reorganization of the KIF network in AEC. Funded by AHA and NIH-NHLBI.

2516

**The Intermediate Filament Protein, Synemin, Anchors Protein Kinase A at the Sarcolemma and Z-lines of Cardiac Myocytes**

L. M. Lund,[1] M. Russell,[2] R. Haber,[2] K. McKeegan,[2] N. Cianciola,[2] G. Vives-Rodriguez,[1] M. Bond[1,2]; [1]Physiology, University of Maryland, Baltimore, Baltimore, MD, [2]Dept. of Molecular Cardiology, The Cleveland Clinic Foundation, Cleveland, OH

In cardiac myocytes (CM), stress generated at sarcomeres is transmitted to the extracellular matrix through costameres by the intermediate filament (IF) network. Synemin binds to desmin, vimentin, α-dystrobrevin and vinculin using its amino-terminus, as well as α-actinin and vinculin via its carboxy-terminus. We have shown that synemin is an A-kinase anchoring protein (AKAP). AKAPs regulate cAMP-dependent protein kinase (PKA) distribution by binding the regulatory (RII) subunit of PKA, to target it near substrates. A human heart expression library was screened using phage display, and novel RII-binding peptides were sequenced. Three synemin clones were identified and an RII overlay assay demonstrated that RII bound a FLAG-tagged synemin peptide. Next, we immunoprecipitated RII from adult rat CMs and identified synemin as a co-immunoprecipitating protein. Using immunohistochemical studies with adult rat ventriclar myocytes, we showed colocalization of synemin with RII at the Z-line and perinuclear region. Because several sarcolemmal proteins also bind to synemin, we isolated rat neonatal CMs to examine this region. Synemin localized at the sarcolemma and did not colocalize with desmin, which was filamentous and cytoplasmic. The two isoforms of synemin (α and β) differ by a 932bp deletion in the tail domain. To determine whether changes in localization were related to differential expression of α- or β-synemin, NCMs were transfected with α- or β-synemin plasmids. After 4 days, cultures overexpressing α-synemin were more elongate with processes present, while β-synemin cultures were similar in size and shape to control CMs. α-Synemin cultures showed more intense staining of the sarcolemma and the processes, whereas β-synemin cultures stained Z-lines. We hypothesize that synemin-anchored PKA phosphorylates substrates present at cell adhesion sites and the sarcolemma through localization by α-synemin and at Z-lines through localization by β-synemin.

2517

**Insights into the Role of Kazrin in Mammalian and Amphibian Epithelia**

L. M. Sevilla,[1] A. A. Rana,[2] K. R. Groot,[1] J. C. Smith,[2] F. M. Watt[1]; [1]Keratinocyte Laboratory, Cancer Research UK London Research Insititute, London, United Kingdom, [2]Wellcome Trust/Cancer Research UK Gurdon Institute and Department of Zoology, University of Cambridge, Cambridge, United Kingdom

Kazrin was identified as a binding partner of the cornified envelope precursor protein periplakin and has been found to colocalize with periplakin and desmoplakin at desmosomes and with periplakin at the interdesmosomal plasma membrane. Kazrin is alternatively spliced resulting in N- and C- terminal splice variants. Overexpression of kazrin isoforms results in alterations in the cytoskeleton and in keratinocyte cell shape. We have analysed the role of kazrin in *Xenopus* embryo development. In Xt embryos expression of kazrin is widespread, with highest levels in the head, eyes, neural tube, and somites. Kazrin expression in *Xenopus* embryos was greatly reduced by injection of morpholino-modified antisense oligonucleotides. This reduction of expression results in multiple developmental abnormalities including perturbation of axial elongation and abnormal head, eye and neural tube development. These results indicate that kazrin plays a fundamental developmental role in *Xenopus*.

2518

**Investigating the Regulation of Keratin 17 Gene Expression in Live Mouse Tissue**

D. J. DePianto, N. P. Bianchi, K. McGowan, C. Gu, P. A. Coulombe; Biological Chemistry, Johns Hopkins University, Baltimore, MD

Keratin genes afford, given their large number (>50) and differential regulation, a unique opportunity to study the mechanisms underlying specification and differentiation in epithelia of higher metazoans. Moreover, the small size and regulation in cis of many keratin genes enable the use of their regulatory sequence to achieve targeted gene expression in mice. Here we show that 2 kilobases of 5' upstream region from the mouse keratin 17 gene (mK17) confers expression of green fluorescence protein (GFP) in major epithelial appendages of transgenic mice. Like mK17, onset of {mK17 5']-GFP reporter expression coincides with the appearance of ectoderm-derived epithelial appendages during embryonic development. In adult mice, [mk17 5']-GFP is appropriately regulated within hair, nail, glands, and oral papilla. Tracking GFP fluorescence allows for visualization of growth cycle-related changes in hair follicles, and the defects engendered by the hairless mutation, in live skin tissue. Deletion of an internal 48 bp-interval, which encompasses a sonic hedgehog responsive element, from this promoter results in loss of GFP fluorescence in most appendages in vivo. The compact mK17 gene promoter provides a novel tool for appendageal-preferred gene expression and manipulation in transgenic mice.

2519

**Changes in Cytoskeletal and Nucleoskeletal Intermediate Filament (IF) Expression during the Epithelial/Mesenchymal Transition in Human Embryonic Stem Cells (hESC)**

M. G. Mendez, R. D. Goldman; Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL

Human IF proteins are encoded by ~65 developmentally regulated genes, and while developmental changes in IF expression are hypothesized to reflect functional differences, these changes have been difficult to study. Human ESC provide an excellent opportunity to study the assembly and functions of different types of IF during differentiation. Pluripotent hESC are cuboidal epithelial cells that grow in tightly packed colonies, and they express nuclear lamin B and keratins 8/18. Cuboidal epithelial cells near the edge of a colony frequently acquire a mesenchymal shape and migrate away. In the earliest stages of this epithelial-to-mesenchymal transition (EMT) lamin A, nestin and vimentin are detected. Initially, pre-LA and nestin appear to be diffusely organized in the nucleus and cytoplasm, respectively. Cells close to the edge of colony begin to express vimentin precursor particles and in more distal regions of the colony they begin to exhibit particles and squiggles (short IF) containing both vimentin and nestin. At the same time, mature lamin A is seen in a largely nucleoplasmic speckle pattern. Finally mature vimentin/nestin IF networks appear in the extensively flattened mesenchymal cells that are moving away from the colony. These cells also express a typical lamin A pattern that colocalizes with lamin B. The expression of lamin A also correlates with significant nuclear shape changes. Interestingly, the nestin/vimentin IF network appears to assemble along the keratin IF network. To date, the data suggest that IF networks expressed early in development provide scaffolds or templates which direct the assembly of IF networks later in development. We are in the process of developing methods for employing siRNA and transient transfection to determine the functional significance of these changing expression patterns. Supported by NIGMS and NCI.

2520

**Autophagy Eliminates Keratin-containing Inclusion Bodies in Cultured Cells**

M. Harada,[1,2] D. M. Toivola,[1] B. M. Omary[1]; [1]Medicine, VA Palo Alto Health Care System and Stanford University, Palo Alto, CA, [2]Medicine, Kurume University School of Medicine, Kurume, Japan

Intracellular inclusions are typically composed of abnormal misfolded proteins, and are found in association with various neurodegenerative and liver diseases. The major components of intracellular inclusions are intermediate filament proteins, which are represented by keratins 8 and 18 (K8/K18) in the liver. To clarify the mechanism of hepatocyte inclusion formation in a model system, we exposed the hepatoma cell line Huh7 to proteasome inhibitors and examined the effects of agents that affect protein synthesis and the autophagy-lysosomal pathway. The proteasome inhibitors ALLN, MG132, lactacystine and epoxomicin induce K8/K18-containing inclusions. Formation of the inclusions is reduced by cycloheximide, an inhibitor of protein synthesis, and rapamycin, a stimulator of autophagy. Immunofluorescence staining showed the inclusions to be in proximity to GFP-LC3 (an autophagic vacuole marker), lysosome-associated membrane proteins 1 and 2, and Lyso-Tracker. Ultrastructural analysis confirmed many autophagic vacuoles and electron dense lysosomes in the proteasome inhibitor-treated cells. Removal of the proteasome inhibitors decreased the number of inclusions over time. This decrease was accelerated by rapamycin, but delayed by bafilomycin A1 which inhibits fusion of autophagic vacuoles and lysosomes. In inclusion-containing cells, the Golgi apparatus was fragmented, as visualized using galactosyl transferase, membrin and GM130 staining, and mitochondria clustered around the inclusions, as visualized using DsRed-Mit. Therefore, formation of keratin-containing inclusions in Huh7 cells depends on a balance between the production of misfolded proteins and degradation by the proteasome and autophagy-lysosome pathway. Inclusion formation alters the distribution of mitochondria and the Golgi apparatus, and autophagy plays an important role in inclusion body elimination. Modulation of protein synthesis or the autophagic pathway may offer a novel strategy to treat patients with inclusion-associated diseases.

2521

**Denaturing Temperature Conditions May Underestimate Keratin Mutation Detection by DHPLC**

P. Strnad,[1] T. C. Lienau,[1] G. Tao,[1] N. Ku,[1] T. M. Magin,[2] M. B. Omary[1]; [1]Dept of Medicine, VA Palo Alto Health Care System and Stanford University, Palo Alto, CA, [2]Institute of Physiological Chemistry, University of Bonn, Bonn, Germany

Keratins 8 and 18 (K8/K18) are the major intermediate filament proteins in digestive organs. K8/K18 variants are risk factors for developing end-stage liver disease and may be associated with inflammatory bowel disease and chronic pancreatitis. However, the frequency of K8/K18 variants in American, British, German, and Italian populations varies. For example, two independent studies showed no amino-acid-altering K8/K18 mutations in 256 German patients with liver disorders, whereas 58 of 467 American liver disease patients had mutations. Both studies used the WaveSystem™, which utilizes denaturing high-performance liquid chromatography (DHPLC). We hypothesized that experimental conditions likely contribute to the discrepancy, and tested this hypothesis using previously described K8/K18 variants to optimize the DHPLC conditions under a range of denaturing temperatures. Several variants, including the frequent K8 G61C, I62V and R340H could not be reliably detected when using temperatures suggested by the prediction software, but all these variants were readily detectable at 2°C higher denaturing temperatures. Using optimized temperatures, we tested for K8 exons 1 and 6 variants, where most of the American cohort K8 variants were reported, using available genomic DNA from 151 of the 256 German liver disease patients. We identified 12 exonic and 2 intronic K8 variants: one G61C, two I62V, seven R340H, one E376E, two intronic E6+46 A-to-T, and one novel K8 V380I. Therefore, although DHPLC offers a robust and high throughput means for mutation analysis, assessment of a denaturing temperature range and possible inclusion of control mutants should be considered.

2522

**Keratins Modulate Mitochondrial HMG-CoA Synthase in the Colon**

D. M. Toivola,[1] A. Habtezion,[1] J. Liao,[2] Q. Zhou,[1] E. C. Butcher,[3] M. B. Omary[1]; [1]Dept. of Medicine, VA Palo Alto Health Care System and Stanford University School of Medicine, Palo Alto, CA, [2]Applied Biomics, Inc., Hayward, CA, [3]Dept. of Pathology, VA Palo Alto Health Care System and Stanford University School of Medicine, Palo Alto, CA

Keratin 8 (K8) was the first intermediate filament (IF) protein targeted for deletion in transgenic mice. In addition to a liver injury susceptibility phenotype, K8-null mice develop, within 2-3 weeks of birth, colonic hyperproliferation and colitis with diarrhea that respond to antibiotic therapy, and enterocyte mistargeting of ion transporters. We used a proteomic approach as a handle to better understand the function of keratins in the colon, and the colitis that is induced by their absence. Proteins were isolated from wild-type (WT) and K8-null colonic crypts using calcium-free

conditions and analyzed using two-dimensional differential in gel electrophoresis (2-D DIGE) coupled with mass spectrometry. Equal amounts of isolated crypt proteins were labeled with Cy3 (WT) and Cy5 (K8-null) and the combined samples were separated using 2-D DIGE. In K8-null enterocytes, eleven major nonkeratin proteins were down-regulated which include selenium binding protein, 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) synthase and carbonic anhydrase I. Fourteen major proteins were up-regulated including dimethylglycine dehydrogenase, and argininosuccinate synthase 1. We focused on HMG-CoA synthase, an important mitochondrial regulator of ketogenesis, whose four isoforms were downregulated 3-6 fold in K8-null colonic crypts. The decreased expression of HMG-CoA synthase was confirmed at the protein (by immunoblotting and immunofluorescence staining) and RNA levels. Changes in colonic HMG-CoA synthase were specific to the colon in that they were not found in the liver. Peroxisome proliferator-activated receptor α (PPARα), an HMG-CoA synthase transcriptional activator was also down-regulated in K8-null colon. These results suggest that colonic keratins may be involved in lipid and ketone body metabolism, which is likely relevant to the colitis phenotype since bacteria are the major source of colonic butyrate.

2523

**Alterations in Intermediate Filament (IF) Assembly Modulate Lamellipodial Activity**

B. Helfand,[1] S. Khoun,[1] E. R. Kuczmarski,[1] H. Herrmann,[2] U. Aebi,[3] R. Goldman[1]; [1]Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL, [2]Department of Cell Biology, German Cancer Research Center, Heidelberg, Germany, [3]M.E. Muller Institute Biozentrum, University of Basel, Basel, Switzerland

Growth factors stimulate cell motility by inducing the formation of lamellipodia. This involves organizational changes in microfilaments and microtubules, but the role of IF in this response remains unknown. Therefore, we have studied vimentin IF following the addition of growth factors to serum-starved mouse embryo fibroblasts (MEF). To monitor changes in living cells, MEFs expressing GFP-vimentin were serum-deprived for 2 days. These cells contained complex IF networks extending to their quiescent cell surfaces. Immediately following growth factor addition, the peripheral IF network disassembled as lamellae formed and only highly motile vimentin particles and squiggles were detected in this region. IF network disassembly and the appearance of vimentin particles and squiggles could also be observed by immunofluorescence in non-transfected MEFs treated identically. Vimentin particles were concentrated in the lamellipodial region and colocalized with antibodies against actin associated proteins such as cortactin. The disassembly of IF concomitant with the appearance of vimentin particles and squiggles suggest that IF disassembly modulates lamellipodial formation. To test this possibility further, a specific peptide inhibitor of vimentin assembly was microinjected into serum-starved MEFs which had been transfected with GFP-vimentin. At low concentration in injection buffer, this peptide (residues 355 to 412 of the alpha-helical 2B domain of vimentin), preferentially disassembled long IF adjacent to the cell surface. Coincidently these serum-deprived cells began to ruffle (in the absence of serum) and form lamellipodia, and only vimentin particles and squiggles were detected in these regions. Injected controls remained quiescent. We propose that polymerized IF stabilize the cell surface and that their local disassembly is sufficient to promote the formation of lamellae/lamellipodia. This appears attributable to a local reorganization of insoluble IF subunits, since biochemical studies of serum-replenished cells did not reveal any Triton-soluble vimentin. Supported by NIGMS.

2524

**Co-translational Assembly of Intermediate Filaments (IF): Messenger RNA Clustering May Be Involved in the Formation of Coiled Coils**

L. Chang,[1] Y. Shav-Tal,[2] R. H. Singer,[2] R. D. Goldman[1]; [1]Cell & Molecular Biology, Northwestern University Medical School, Chicago, IL, [2]Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Peripherin IF networks are formed from precursor particles that are most likely composed of essential building blocks such as dimers and tetramers. Using a combination of RNA fluorescence in situ hybridization (FISH) and the simultaneous live imaging of both peripherin mRNA and its product in PC12 cells, we have shown that ~35% of peripherin particles are associated with peripherin mRNA in a puromycin sensitive fashion. There is no significant association with other species of mRNA such as actin. Quantitative analyses demonstrate that individual peripherin mRNA containing particles (mRNPs) can contain up to 30 copies of transcript. Microtubules appear to be involved in mRNA clustering as nocodazole markedly decreases the number of clusters. To determine the potential role of peripherin nascent protein in clustering, a translation incompetent peripherin mRNA was constructed. In the absence of nascent protein, the majority of mRNA still forms clusters. However nascent peripherin appears to be involved in the formation of large RNA clusters (6-30). We are currently examining the specific role of the peripherin 3'UTR in mRNA clustering. We have also initiated studies on keratin IF whose building blocks are heterodimers. A sub-population of keratin 8 and 18 mRNAs in Hela cells appear to co-exist in common mRNPs further suggesting that IF precursor particles are formed co-translationally. The formation of a coiled coil has been traditionally believed to involve a polysome containing a single transcript. The asynchronous translation in a polysome would give rise to protein chains of varying lengths that complicates in-register dimer formation. Coiled coils may be more efficiently formed from peptide chains that are being simultaneously synthesized at the same rate from neighboring mRNAs within a translation factory. Supported by NIGMS

2525

**Insights into the Effects of the Common Lamin A Mutation in Hutchison-Gilford Progeria Syndrome (HGPS)**

T. Dechat,[1] S. A. Adam,[1] M. R. Bozovsky,[1] A. E. Goldman,[1] D. K. Shumaker,[1] A. Rusinol,[2] M. S. Sinensky,[2] R. D. Goldman[1]; [1]Cell and Molecular Biology, Northwestern University, Feinberg Medical School, Chicago, IL, [2]Biochemistry and Molecular Biology, East Tennessee State University, JH Quillen College of Medicine, Johnson City, TN

HGPS is a premature aging disease commonly caused by a heterozygous mutation in the lamin A (LA) gene leading to a deletion of 50 residues at the C-terminus of LA (LAΔ50). Pre-LA is farnesylated and methylated at its C-terminal CAAX box. The carboxyl terminal 15 amino acids are then removed by a second cleavage step producing LA. Since LAΔ50 lacks the second cleavage site it may be stably farnesylated/methylated. This study shows that this is indeed the case. LAΔ50 is shown to be processed at the CAAX-box to approximately the same extent as lamin B. This results in LAΔ50 being associated with the nuclear lamina/membrane as opposed to the nucleoplasmic localization of wild type (wt) LA. Microscopic studies of HeLa cells expressing GFP-LAΔ50 or HGPS patients' cells reveal that LAΔ50 localizes exclusively to the nuclear envelope throughout interphase. At the same time, nucleoplasmic wt LA disappears and it is only detected in the lamina region. During mitosis LAΔ50 stays membrane associated in contrast to wt LA, which is soluble and dispersed throughout the cytoplasm. During early G-1 there is a delayed reentry of

LAΔ50 into daughter cell nuclei. Cell fractionation studies of mitotically arrested cells demonstrate that LAΔ50 is membrane associated in contrast to wt LA. In conclusion, LAΔ50 is stably associated with the nuclear membrane. This appears to recruit wt LA to the nuclear periphery causing a depletion of LA from nucleoplasmic complexes involved in cell cycle regulation and other activities causing loss of functions in HGPS. Supported by NIA, PRF and the Ellison Foundation.

2526

**Keratin Mutation Predisposes to Thioacetamide- but not CCl4-induced Mouse Liver Fibrosis**

P. Strnad, Q. Zhou, N. Ku, M. B. Omary; Department of Medicine, VA Palo Alto Health Care System and Stanford University, Palo Alto, CA

Keratins 8 and 18 (K8/K18) protect mouse liver from a variety of stresses, and K8/K18 mutations predispose their human carriers to end-stage liver disease. Since the relationship of keratin mutation to cirrhosis is based on human association studies, we sought to provide direct evidence for a keratin mutation-related predisposition to liver fibrosis using mouse models. We compared the development of liver fibrosis in mice overexpressing human Arg89-to-Cys (R89C mice) or wild-type K18 (WT mice). Liver fibrosis was induced by intraperitoneal injection of carbon tetrachloride or thioacetamide for 6-8 weeks. Expression of fibrosis-related genes was measured by quantitative real time PCR, and trichrome or Sirius red staining were used to evaluate the extent of fibrosis. CCl4 led to liver and hemorrhagic lung injury but there were no differences in the extent of CCl4-induced liver fibrosis between WT and R89C mice when accessed by histological staining and quantitative real time PCR. In contrast, thioacetamide caused a more severe fibrosis in R89C as compared to TG2 mice as determined by histological staining and by increased levels of collagen, TIMP1, MMP2, MMP13 mRNA. Therefore, overexpression of mutant K18 predisposes transgenic mice to thioacetamide- but not CCl4-induced liver fibrosis. Differences in the keratin mutation-related response to the thioacetamide versus the CCl4 model raise the hypothesis that keratin mutations may preferentially predispose to fibrosis in unique human liver diseases.

2527

**Keratins Modulate Hepatocyte Mitochondria: A Likely Contributor To Keratin Cytoprotective Function**

G. Tao,[1] D. M. Toivola,[1] J. Liao,[2] B. Liang,[1] M. B. Omary[1]; [1]Medicine, Palo Alto VAMC and Stanford University, Palo Alto, CA, [2]Applied Biomics Inc, Hayward, CA

Keratin polypeptides 8 and 18 (K8/K18) mutations are a risk factor for developing human end-stage liver disease. Studies in K8-null and K18 mutant-expressing mice showed that K8/K18 mutation or absence predisposes hepatocytes to apoptosis and consequent liver injury and primes them towards oxidative damage. We addressed the molecular mechanism for such predispositions using proteomic two-dimensional differential in gel electrophoresis (2-D DIGE) coupled with mass spectrometry to analyze changes in livers isolated from K8-null and control mice. Among the most prominent alterations noted in the total liver homogenates were in mitochondria-specific proteins and their charged isoforms, which were verified by 2-D DIGE of mitochondria preparations isolated from K8-null and wild-type livers. The altered proteins in K8-null versus control hepatocytes include those involved in ATP-producing respiratory chain at the mitochondrial inner membrane (e.g. an increase in NADH-ubiquinone oxidoreductase 75 kDa subunit of complex I), those involved in apoptosis (e.g. a decrease in cytochrome c content of mitochondria) and those that serve an antioxidant role (e.g. a decrease in mitochondrial superoxide dismutase-2). Ultrastructural analysis showed that mitochondria of K8-null livers were rounder and significantly smaller as compared with mitochondria from WT livers. Therefore, the mechanical and nonmechanical roles of keratins in regulating hepatocyte function may extend to similar roles in regulating the shape and function of mitochondria. Loss of keratin function via mutation or ablation and the consequent effects on mitochondria are likely to contribute to hepatocyte predisposition to apoptosis and oxidative injury.

2528

**Localization of Keratin Intermediate filaments, Fas-receptor and Phospho-Akt in Griseofulvin Treated Hepatocellular Carcinoma Cells HepG2**

A. Fortier, É. Asselin, M. Cadrin; Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, PQ, Canada

Keratin 8 and 18 (K8/18) are the intermediate filament (IF) proteins present in hepatocytes. IFs protect hepatocytes from mechanical and toxic injuries as demonstrated by studies using different genetically altered mouse models. For instance, treatment of K8 null mice with a diet containing griseofulvin (GF) is highly toxic and causes animal death by liver failure. The same treatment on wild type mice is not fatal and induces modifications, in hepatocytes, of K8/18 expression, phoshorylation and in the long-term formation of K8/18 containing aggregates named Mallory bodies. The aim of the present study was to gain insight into the molecular mechanism underlying keratin protective role in hepatocytes. Hepatocellular carcinoma cells HepG2, were treated with GF (0.2µg/ml in 0.1% DMSO) for 8h, 24h and 72h. We investigated the existence of a relationship between K8/18 IFs, and the serine/threonine kinase Akt/PKB (pAkt) which plays a pivotal role in cell survival. We also analyzed Fas an important inducer of the extrinsic apoptosis signaling pathway. Our results show that in control HepG2 cells, K8/18 forms a filamentous network that extend from the nucleus to the cell periphery. Fas and pAkt are diffusely distributed in the cytoplasm of the cells with some accumulation in the Golgi apparatus. GF treatment induces Fas rearrangement and its re-localization to the plasma membrane. Fas form also cytoplasmic filamentous structures that co-localize with K8/18 IFs. Staining of pAkt is increased after treatment and as for Fas, co-localization of pAkt and K8/18 IFs is observed. Together, these results suggest that keratins could accomplish their protective roles by modulating specific signal transduction pathways through their association with regulatory molecules such as Akt and Fas.(Supported by NSERC)

2529

**Molecular "Memory" via Chaperone Modification as a Potential Mechanism for Rapid Mallory Body Induction**

P. Strnad,[1] J. Liao,[2] K. Lau,[3] J. Schilling,[3] M. B. Omary[1]; [1]Dept of Medicine, VA Palo Alto Health Care System and Stanford University, Palo Alto, CA, [2]Applied Biomics Inc, Hayward, CA, [3]Department of Pediatrics, Stanford University School of Medicine, Palo Alto, CA

Cytoplasmic inclusions termed Mallory bodies (MB) are characteristic of several liver disorders including alcoholic and non-alcoholic steatohepatitis. In mice, MB can be induced by long-term feeding with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 3-4 months or rapidly re-formed in DDC-induced then recovered mice by DDC re-feeding for 5-7 days. We hypothesized that molecular changes are likely to be retained after DDC priming, which may explain the rapid re-induction of MB and may contribute in general terms to MB formation. We tested this

hypothesis using two-dimensional differential in gel electrophoresis (2-D DIGE) coupled with mass spectrometry to characterize changes in liver proteins isolated from the various treatment groups. Tissue lysates from different mice were labeled with Cy3 or Cy5 and analyzed by 2-D DIGE. Image analysis was performed with ImageQuant V5.0 and DeCyder 4.0 software. Proteins representing individual spots were identified using MALDI-TOF analysis. DDC treatment led to pronounced charged isoform changes in several chaperones including Hsp 25, 60, 70, 72, 75, GRP58, GRP75 and GRP78, which lasted at least for 1 month after discontinuation of DDC feeding. Furthermore, recovered mice had persistent isoform changes in several mitochondrial proteins (GRP75, ALDH4A1, Hsp70, GST Mu). Posttranslational modifications that can account for such changes included arginine and lysine oxidation, asparagine and glutamine deamidation, and methylation of several residues. We conclude that persistent posttranslational modifications in chaperone proteins with possible mitochondrial dysfunction likely contribute to the rapid MB formation, and provide a form of molecular "memory". We hypothesize that chaperone dysfunction via oxidation and other posttranslational modifications is an important contributor to inclusion body formation in several diseases.

2530

**New Dimensions in Intermediate Filament Dynamics: Quantitative Analysis of Vimentin Speckles by Cross Correlation of Intensity Profiles Reveals Non-random Movement of Speckles Along Individual Fibers**

L. Chang,[1] S. Sivaramakrishnan,[1] C. M. Waterman-Storer,[2] T. Wittmann,[2] R. D. Goldman[1]; [1]Cell & Molecular Biology, Northwestern University Medical School, Chicago, IL, [2]Cell Biology, The Scripps Research Institute, La Jolla, CA

GFP-vimentin intermediate filaments (IF) appear speckled when expressed at low levels in Ptk1 cells. Unlike the coordinated treadmilling of kinetochore fiber speckles and the sparkling of actin speckles in leading edges of cells, vimentin speckles display a remarkable variety of motile properties. These include tandem movements of linear speckle arrays at varying speeds as well as independent movements of speckles toward or away from each other, possibly within a single filament or filament bundle. The various types of speckle movements may represent whole filament movements, subunit movements within a filament, or the sliding movements of filaments within bundles. In addition, some of these movements could be attributed to non-filamentous IF precursor particles moving along side an existing filament. Particle tracking of individual speckles is not feasible as movement along apparent filament paths occurs in short bursts with the filaments themselves drifting and changing shape within the cytoplasm. We have developed a novel technique to analyze the apparent coordinated movement of IF speckles by examining the cross correlation coefficients of intensity profiles along filaments between time frames. This analysis allows us to quantify speckle velocities and displacements and is providing insights into the molecular mechanisms that contribute to speckle movement. Analysis of intensity fluctuations of speckles by correlating the intensity distributions of several speckles suggests bidirectional movement along relatively straight paths with varying velocities, strongly suggesting either motor directed movement or wave propagation along individual filaments. This is the first study to quantitatively explore speckling of IF in live cells and reveals fascinating dynamics underlying IF networks. Funded by NIGMS AND AHA

2531

**Colitis-Associated Autoantibody Production in Keratin-8 Deficient Mice**

D. M. Toivola,[1] A. Habtezion,[1] L. Y. Zhang,[1] W. H. Robinson,[1] P. J. Utz,[1] E. C. Butcher,[2] M. B. Omary[1]; [1]Dept of Medicine, VA Palo Alto Health Care System and Stanford University Digestive Disease Center, Palo Alto, CA, [2]Dept of Pathology, VA Palo Alto Health Care System and Stanford University Digestive Disease Center, Palo Alto, CA

Keratin 8 (K8)-null mice develop an early-onset intestinal phenotype with colorectal hyperplasia, colitis with diarrhea, mistargeting of enterocyte ion transporters as well as increased susceptibility to liver damage. Several human autoimmune diseases, such as inflammatory bowel disease, are associated with autoantibody production which led us to hypothesize that K8-null mice may produce such antibodies. To test this hypothesis, we analyzed whether sera from wild-type and K8-null mice recognize autoantigens by immunoblotting total tissue lysates of normal mouse liver, pancreas and colon as well as lysates of human/mouse cell lines of various origins. We identified one major autoantibody that was present in six of eleven (55%) sera from K8-null mice but not in wild-type sera. Presence of the autoantibody is age and disease severity related since the six positive K8-null sera were from mice >7 months old. The autoantibody recognizes a ubiquitous 42-48 kD autoantigen that partitions in a non-ionic detergent fraction, and is present in all normal tissues and cell lines tested, as well as in livers and colons of K8-null mice. Immunofluorescence staining of NIH-3T3 cells showed that the autoantigen concentrates in cytoplasmic and perinuclear structures but not the nucleus or plasma membrane. Our data support the hypothesis that K8-null colitis is associated with age-enhanced autoantibody production, which also associates with a worsening colonic disease. The 42-48 kDa ubiquitous autoantigen is cytoplasmic and detergent soluble, and is conserved across human and mouse species. Characterization of this antigen may unmask a similar autoantibody response in patients with inflammatory bowel disease who harbor K8 mutations.

## Extracellular Matrix & Cell Behavior II (2532-2546)

2532

**Encapsulation of Human Mesenchymal Stem Cells in a 3D Extracellular Matrix Environment for the Direction of Osteogenesis**

A. W. Lund,[1] E. P. Lunsford,[1] G. Lin,[2] B. Roysam,[2] J. P. Stegemann,[3] G. E. Plopper[1]; [1]Biology, Rensselaer Polytechnic Institute, Troy, NY, [2]Electrical, Computer, & Systems Engineering, Rensselaer Polytechnic Institute, Troy, NY, [3]Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY

Adult human mesenchymal stem cells (hMSC) have demonstrated potential for osteogenesis and their ability to self replicate and differentiate along this pathway is significant within the field of functional tissue engineering. Two dimensional cultures alone are insufficient for the study of such processes as well as incomplete in terms of application to natural systems. A method of encapsulating these cells within a controlled extracellular matrix (ECM) has been developed resulting in the production of distinct spherical microenvironments. These "beads" have a defined size and composition that can be manipulated to help induce and maintain a desired cellular phenotype. It is proposed that controlled manipulations of the structure and composition of the ECM beads will create an osteoconductive matrix and produce more robust and natural bone formation as compared to traditional methods alone. These changes are monitored through biochemical assays, immunofluorescence and novel image analysis technologies to yield a predictive model for the path of osteogenic differentiation. Calcification, mineralized matrix deposition, protein expression,

and gene expression provide the markers to define differentiation. Fluorescence images, along with analysis software, provide a new way of characterizing and quantifying cellular response within the 3D environment that allows for the preservation of spatio-temporal relationships. It is proposed that there is a correlation between the information gathered through traditional biochemical assays and that gathered by imaging software which can be exploited to create a new and easier approach to functional tissue engineering. This ability to control the ECM environment surrounding the cells, while assessing the functional state of the cells in intact constructs, should have broad application to studies of cell growth and differentiation.

2533

**Physiological Activities of Human Fetal Osteoblast Cells Cultured on Gamma Titanium Aluminide Determined by Immunoassays of Collagen Type I and Osteonectin Proteins**

O. Rivera-Denizard,[1] N. Diffoot-Carlo,[1] V. Navas,[1] P. Sundaram[2]; [1]Biology, University of Puerto Rico-Mayaguez, Mayaguez, PR, [2]Mechanical Engineering, University of Puerto Rico-Mayaguez, Mayaguez, PR

Commercially pure (cpTi) and titanium alloys, such as Ti-6Al-4V, are among the most common used implant materials. There is a continuous search for new materials for use in implants with better biocompatibility properties than the current cpTi and Ti-6Al-4V alloys. Gamma titanium aluminide (γTiAl), a new titanium alloy originally designed for aerospace applications, appears to have better properties compared to Ti-6Al-4V. The biological response to γTiAl implant is expected to be similar or better than those of Ti-6Al-4V. hFOB 1.19 cells (ATCC CRL-11372) were cultured at 37C on 1 cm diameter discs of γTiAl and Ti-6Al-4V, with variable roughness. Metals were removed after incubation periods of 14, 21 and 28 days and immunohistochemistry analysis was performed in order to detect the presence of collagen type I and osteonectin, proteins of the bone extracellular matrix. Normal growth and the presence of collagen type I and osteonectin was observed for cells cultured on both metals, with better results for γTiAl. The use of γTiAl does not appear to affect the biological activity of the osteoblast cells.

2534

**Coordinate Regulation of Pre-Osteoblastic MC3T3-E1 Cell Phenotype by ECM Chemistry and Mechanics**

C. B. Khatiwala,[1] A. J. Putnam[2]; [1]Chemical and Biochemical Engineering, University of California, Irvine, Irvine, CA, [2]Chemical and Biochemical Engineering, Biomedical Engineering, University of California, Irvine, Irvine, CA

Mechanical cues present in the extracellular matrix have been hypothesized to provide instructive signals that dictate cell behavior, and may be equally as important as chemical information. We probed this hypothesis in the context of bone by culturing pre-osteoblastic MC3T3-E1 cells on the surface of ECM-functionalized hydrogels with tunable mechanical properties. On gels functionalized with low density of type-I collagen, the rate of cell proliferation increased linearly with increasing stiffness. Quantitative time-lapse video microscopy analysis revealed random cell motility speeds were significantly retarded on the softest substrate (0.25 ± 0.05 μm/min), in contrast to maximum speeds on polystyrene substrates (0.42 ± 0.04 μm/min). Immature focal contacts and a poorly organized actin cytoskeleton were observed in cells cultured on the softest substrates, in contrast to mature focal adhesions and robust actin stress fibers on more rigid substrates. In parallel, focal adhesion kinase activity was modulated by ECM rigidity, with maximum Y397-FAK levels observed in cells cultured on polystyrene. When gels were functionalized with a high density of collagen-I, migration speed was found to depend on ECM compliance in a biphasic manner, with maximum speeds (0.36 ± 0.04 μm/min) observed on gels of intermediate stiffness and minimum speeds (0.24 ± 0.04 μm/min) occurred on both the softest and most rigid (i.e., polystyrene) substrates. In parallel, focal adhesion-associated vinculin and Y397-FAK levels were significantly increased on the soft substrates, but remained unaltered by ECM ligand density on stiff substrates. Finally, mineral deposition by the cells was also modulated by ECM compliance as early as day 4 in culture. These results suggest that tuning ECM chemistry and mechanics, which presumably coordinately influence the degree of actin contractility, can regulate focal adhesion formation, signaling, migration, proliferation, and, ultimately, differentiation towards an osteoblastic phenotype.

2535

**Tenascin-C Plays a Crucial Role in Progression of Myocardial Fibrosis**

T. Nishioka, M. Suzuki, T. Yoshida, K. Imanaka-Yoshida; Pathology, Mie University, Tsu, Japan

Tenascin-C (TN-C) is an extracellular glycoprotein with strong bioactivity to control cell behavior. In the normal heart, tenascin-C is detected only at very early stage of embryogenesis but re-expressed in the various heart disease. During scar formation after myocardial injury, TN-C deposition precedes maturation of collagen fibers and is supposed to play important roles in progression of fibrosis. To elucidate the role of TNC in myocardial fibrosis in hypertensive heart, we treated mice. Balb/c wild type (WT/AgII) mice and TNC KO (TNKO/AgII) mice were treated with 500 ng/kg body weight/min AgII subcutaneously by osmotic minipump for 4 weeks. In both wild type and TNKO mice, AgII treatment increased blood pressure, and heart weight/Body weight ratio, but no significant differences were detected between WT/AgII and TNKO/AgII mice. AgII treatment caused increase of interstitial collagen fibers increased at perivascular regions. In KO/AgII, increase of interstitial collagen fibers in was less than those in WT/AgII. Accumulation of macrophages and myofibroblsts were evaluated by immunohistochemistry staining with either antibody anti Mac-3 or anti alpha-SMA, respectively. In WT/AgII mice, accumulation of macrophages and myofibroblasts aobserved at peivascular region. accumulation of macrophages and myocfiroblasts were reduced in TNKO/AgII. Alcian Blue staining showed dense deposition of glycosaminoglycans (GAGs) at perivascular area in WT/AgII. In contrast, TNKO/AgII showed sparce and rough fibrillar staining pattern. These results suggest that, TNC plays important roles in progression of fibrosis in hypertensive heart.

2536

**Fibroblast Mechanics in Three Dimensional Collagen Matrices: Microtubules Take the Load**

S. Rhee, F. Grinnell; Department of Cell Biology, UT Southwestern Medical Center, Dallas, TX

The interaction between cells and three dimensional collagen matrices provides a useful model to study cell physiology under conditions that approximate normal tissue better than conventional 2D culture. A variety of evidence shows that important aspects of cell form and function differ in 3D matrices compared to 2D culture. Mechanisms underlying these differences, however, remain speculative. The tensegrity hypothesis of cell mechanics predicts that the balance between structures that resist compression (e.g., microtubules) and structures under tension (e.g., actin

filaments) determines cell organization. We tested the role of microtubules in cell mechanics using human fibroblast-3D collagen matrix cultures. In contrast to cells on collagen-coated coverslips, fibroblasts interacting with 3D collagen matrices became completely dependent on intact microtubules for mechanostabilization. If microtubules were disrupted, then cells could not change shape or migrate. The influence of the collagen matrix on microtubule-dependence of cell mechanics depended on the physical properties of the cell-matrix interface rather than on cells becoming completely surrounded by matrix. Only if contractile tension in the actin cytoskeleton was blocked, were cells then able to protrude dendritic extensions without intact microtubules. Our studies suggest that fibroblasts in 3D collagen matrices utilize different mechanisms of mechanical stabilization compared to cells in conventional 2D culture. The rigid culture surface can provide external mechanical resistance that balances tension in the actin filament network and, as a result, decrease the importance of microtubules for cell organization and migration. In 3D matrix cultures, on the other hand, in the absence of a rigid, planar culture surface, microtubules take the mechanical load as predicted by tensegrity. These observations emphasize that at the fundamental level of cytoskeletal organization, regulation of cell mechanics depends on the physical interface between cells and their environment.

2537

**The Intrinsic Mechanical Properties of the Extracellular Matrix Regulate Smooth Muscle Cell Phenotype**

S. R. Peyton,[1] C. B. Raub,[2] A. J. Putnam[2]; [1]Chemical Engineering and Materials Science, University of California, Irvine, Irvine, CA, [2]Biomedical Engineering, University of California, Irvine, Irvine, CA

An emerging paradigm in cell biology hypothesizes that intrinsic mechanical cues present in the extracellular matrix (ECM) may be equally important as its chemical identity in regulating tissue development. In a variety of cardiovascular pathologies, the mechanical properties of blood vessels may be dramatically altered due to cholesterol deposition, changes in fluid shear, or changes in blood pressure. These local mechanical cues can affect smooth muscle cell (SMC) migration, proliferation, and matrix deposition within arteries. To investigate the influence of changing ECM mechanics during these pathologies, we have adopted synthetic model systems from polyacrylamide and poly(ethylene glycol) (PEG) that contain tunable mechanical properties and can support cell adhesion via functionalization with native, full-length proteins and bioactive peptide sequences. Specifically, using 2-D polyacrylamide substrates, we found that the migration speed of SMCs depends on ECM mechanics in a novel, biphasic fashion, suggesting the existence of an optimal substrate stiffness capable of supporting maximal migration. The value of this optimal stiffness shifted depending on the concentration of ECM protein covalently attached to the substrate. Additionally, this phenomenon appears to be controlled by the RhoA-ROCK pathway as blocking ROCK activity via Y27632 retards the maximum migration speed of SMCs on optimally stiff substrates. To investigate the long-term effects of substrate mechanics on the phenotypic conversion of SMCs from a contractile to synthetic phenotype in a 3-D environment, we have begun to use a PEG-based biomaterial system in which the surrounding substrate mechanics and ligand density presented to the cell can be independently controlled. Combined, our results demonstrate the utility of synthetic hydrogel systems to decipher how ECM chemistry and mechanics coordinately regulate SMC phenotype, and that the use of synthetic ECM analogs can contribute to our understanding of cardiovascular disease.

2538

**The Role of Rho-kinase in Local and Global Cell-induced 3-D Matrix Remodeling**

A. Kim, W. M. Petroll; Department of Ophthalmology, UT Southwestern Medical Center, Dallas, TX

The purpose of this study was to investigate the role of Rho-kinase on the pattern and amount of cell-induced collagen matrix remodeling. Human corneal fibroblasts were plated at low density inside 100 μm thick fibrillar collagen matrices and cultured for 1 day in serum-containing media (S+) and S+ with the Rho-kinase inhibitor Y-27632. Cells were then fixed and stained with rhodamine phalloidin. Fluorescent (for f-actin) and reflected light (for collagen fibrils) 3-D optical section images were acquired using laser confocal microscopy. Fourier transform analysis was used to assess local collagen fibril alignment, and changes in cell morphology and collagen density were measured using MetaMorph. Culture in S+ induced significant global matrix contraction, which was inhibited by blocking Rho-kinase ($44.2 \pm 2.1\%$ vs. $24.3 \pm 3.5\%$, $p < 0.05$). Local collagen fibril density was greatest at the ends of cells in S+, and these fibrils were aligned nearly parallel to the pseudopodial tips ($|\Delta\theta| = 5.0 \pm 6.3°$, where $\Delta\theta$ is the difference between collagen and pseudopodial alignment). Fibril alignment was more random adjacent to the cell body ($|\Delta\theta| = 45.9 \pm 25.1°$, $p<0.05$). Both the fibril density and co-alignment with pseudopodia were significantly reduced by blocking Rho-kinase ($p < 0.05$). Cells also had fewer stress fibers and a more stellate morphology following Rho-kinase inhibition; and the height of the cells (extension of processes along z-axis) was significantly increased ($28.4 \pm 3.5$ μm vs. $15.9 \pm 4.2$ μm, $p < 0.05$). The data suggest that force generation by pseudopodia leads to local compaction and alignment of collagen fibrils, and that this mechanism underlies global matrix contraction. This process is dependent, in part, on Rho-kinase.

2539

**Fibroblast Shape and Organization Changes within Contracting Load Bearing Collagen Gels**

B. Johnson-Wint, B. Coon; Department of Biological Sciences, Northern Illinois University, DeKalb, IL

We are interested in physical organization of collagen fibers by fibroblasts and osteocytes during dense connective tissue and bone formation, and the role of tissue load on cell function. In the present study we examined fibroblast filopodia formation and cell-cell interaction during reorganization of type-I collagen in weight bearing cell populated collagen gels (CPCGs). To track cell bodies and filopodia, isolated rat tendon fibroblasts were stained in vitro with Cell Tracker Green or Cell Tracker Orange (Molecular Probes). Stained cells were mixed and seeded into type I collagen gels. CPCGs were placed under load and imaged live at multiple times with a Zeiss laser scanning confocal microscope. Data stacks were analyzed using Image J. An extensive interconnected network of filopodia formed between fibroblasts in contracting CPCGs. By 4 hours cells had extended multiple, frequently branched, thin 9 micron long filopodia. By 16 hours these filopodia were 3 to 5 times longer and often in point or lateral contact with filopodia of adjacent cells. By 36 hours the cells were closer together and had shorter and thicker filopodia with a larger surface of contact between them. Finally, by 55 hours cells had aligned into long branching strings of laterally adherent cells within a now highly compact CPCG. In conclusion, we found that early in collagen gel contraction fibroblasts extend multiple thin filopodia that attach to each other forming a tight filopodial network. Later, cells adhere side-by-side forming a network of branching cell strings. These two types of network appear to be under tension and may represent two mechanisms whereby cells squeeze fluid out of collagen gels and compact the collagen fibers into denser

stronger tissue of a particular shape.

2540

**Regulation of Fibronectin Receptor Engagement by Manipulating Cell-binding Site Availability in a 3-D Matrix**

Y. Mao, J. E. Schwarzbauer; Molecular Biology, Princeton University, Princeton, NJ

The extracellular matrix protein fibronectin (FN) plays an important role in regulating cell adhesion and migration through interactions with integrin receptors. Many cells express both α5β1 and αvβ3 integrins; α5β1 requires the RGD cell binding sequence along with the synergy site for maximal binding to FN, whereas αvβ3 requires only RGD. This difference in binding sites may provide a mechanism for matrix regulation of receptor usage by cells. To address this possibility, we manipulated the receptor binding sites in FN and tested the effects on cell migration in a 3-dimensional (3-D) matrix. Migration of CHO(B2) cell lines expressing one or both of these integrins was examined on 3-D matrices containing wild type and mutant FNs. CHOα5 cells showed decreased cell adhesion, spreading, and migration on 3-D matrix containing FN(RGD-) which lacks the RGD sequence. Similarly, incorporation of FN(syn-) lacking the synergy site caused a reduction in CHOα5 cell migration. In contrast, the absence of the synergy site had no effect on migration of CHOαvβ3 cells. Interestingly, CHOα5/αvβ3 cells, which express both integrins at similar levels, selectively used α5β1 to mediate migration on wild type FN matrix but used αvβ3 integrin for migration on FN(syn-) matrix, as shown using specific function-blocking antibodies. To determine whether αvβ3 and α5β1 integrins used different intracellular machinery, we isolated and analyzed the pseudopodia extended by CHOα5 and CHOαvβ3 cells on FN. The signaling and cytoskeletal proteins FAK, Src, p130CAS and ERM were present at similar levels, but paxillin and activated FAK were significantly enriched in CHOα5 cell pseudopodia. These results show selective usage of FN receptors and downstream signals depending on the availability of binding sites within the extracellular matrix.

2541

**Effects of Mechanical Stimulation on Gene Expression of Chondrocytes Embedded in 3D Hydrogel; the Comparison among Different Modes of Stimulation**

Y. Hirano,[1] N. Ishiguro,[1] M. Sokabe,[2,3] K. Naruse[2,4]; [1]Department of Orthopaedic Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Department of Physiology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [3]Department of Molecular Physiology, National Institute for Physiological Science, Okazaki, Japan, [4]Institute for Advanced Research, Nagoya University, Nagoya, Japan

Articular cartilage is a tough, elastic tissue that covers the ends of bones, enables the bones to move smoothly over one another, and is always subjected to mechanical stress. To study the effects of mechanical stress on the chondrocytes, various kinds of experimental methods including 3-dimensional (3D) culture have been developed. The objective of this study is to develop novel experimental methods to load cells embedded in 3D hydrogel with different modes of mechanical stimulation and to compare gene expressions of chondrocytes embedded in 3D hydrogel among four kinds of mechanical stimulation. The modes of mechanical stimulation used were dynamic strain (10% elongation, 50 cycles/min), 10% static strain, dynamic compression (10% compression, 50 cycles/min), and 10% static compression. Unstimulated control was made at the same time. Mechanical stimulations were loaded to articular chondrocytes from NZW rabbit embedded in collagen gel for strain experiments and agarose gel for compression experiments. After stimulation for 24hours, total RNA was extracted and reverstranscripted. Gene expression of type2 collagen and aggrecan were quantified using real-time PCR. GAPDH was used as endogenous control. Dynamic compression significantly increased expression of type2 collagen and aggrecan compared with control, However, static compression significantly decreased expression of aggrecan and had no significant effect on expression of type2 collagen compared with control. Dynamic strain significantly decreased expression of type2 collagen and aggrecan compared with control, However static strain had no significant effects on both expressions compared with control. These results suggest that dynamic compression has an important role in healthy homeostasis of articular cartilage and static compression or dynamic strain leads to degradation of articular cartilage and that alls may use different mechanotransduction pathway to sense these modes of mechanical stimulations.

2542

**Nanoscale Topographic Features that Mimic the Native Basement Membrane Modulate Vascular Cell Behaviors**

S. J. Liliensiek,[1] P. F. Nealey,[2] C. J. Murphy[1]; [1]Surgical Sciences, University of Wisconsin, Madison, WI, [2]Department of Chemical and Biological Engineering, University of Wisconsin, Madison, WI

Basement membranes have many features including a complex three dimensional topography which influences cell behaviors. The majority of studies investigating the effects of topographic cuing on cells have been conducted on large scale (>1 micron) features, which does not accurately represent the range of feature sizes present in the basement membrane. We have recently conducted morphological analysis of the architecture of the basement membrane underlying the vascular endothelium from aorta, carotid and saphenous vessels from the Rhesus Macaque using SEM. We found that the average pore diameter (62$\pm$37 nm) and fiber size (30$\pm$11 nm) was similar across vessel types and to feature sizes described for the basement membrane of the porcine aortic valve (Brody et al, In Press). Based on the morphological characterization, silicon and polyurethane surfaces with feature sizes mimicking the native basement membrane were produced. The anisotropic surfaces were composed of ridges and grooves containing features ranging from 200 nm to 2000 nm. These surfaces were used to characterize the impact of topographic cueing on endothelial behaviors including orientation/elongation and proliferation. HUVEC and HMVEC cells, representative of endothelial cells from large and small vessels, respectively, were plated onto substrates at a density of 9,000 cells/cm$^2$ and allowed to adhere for 24 hours, fixed , stained and analyzed for orientation/elongation. We found that both HUVECS and HMVECS exhibit alignment and elongation with the topography (40% or more) compared to planar surfaces (10%). At 5 days post plating, proliferation of HUVECS was inhibited by topographic features of widely varying pitch (pitch=ridge+groove) while proliferation of HMVECs was unaffected by topographic cues. Our data indicate a heterogeneous response of endothelial cells to topography and suggest that endothelial cells from large and small vessels respond differentially to topographic cues.

2543

**Probing Cellular Responses to Extracellular Signals through Direct Writing of Proteins with a Microfabricated Patterning Tool**

J. Werbin,[1] C. Lemmon,[2] G. Ledung,[3] W. F. Heinz,[1] L. H. Romer,[4] J. H. Hoh[1]; [1]Physiology, Johns Hopkins University School of Medicine,

Baltimore, MD, [2]Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, [3]Mälardalen University, Eskilstuna, Sweden, [4]Anesthesiology & Crit Care Med, Johns Hopkins University School of Medicine, Baltimore, MD

Interactions between cells and the extracellular environment play a central role in a number of biological phenomena such as tissue growth, cell differentiation and migration. Patterning biomolecules at high resolution on solid supports is a powerful means to investigate how cells interpret and respond to spatially defined extracellular cues. We used a new technology based on microfabricated surface patterning tools, which delivers a protein solution ($10^{-15}$-$10^{-18}$ liters) to the surface via a micro-channel in small cantilever (NanoArrayer, Bioforce Nanosciences). This direct writing process transfers proteins to the substrate by capillary action when the tool touches the surface for times ~100 ms. Proteins can be bound to the surface by adsorption, or via covalent chemistry. The resulting feature sizes range from submicrometer to tens of micrometers, and pattern dimensions are typically 0.5 x 0.5 mm. A series of patterning parameters and conditions have been optimized, and we have constructed glass substrates with a number of different proteins and peptides in complex patterns with multiple components. For example, patterns that are composed of fibronectin and vitronectin where the distance between the two molecules is subcellular, such that a single cell can contact both fibronectin and vitronectin at several well-defined points, have been produced. These patterns have been characterized by immunofluorescence, and are stable under cell culture conditions. In experiments with Mouse Embryo Fibroblasts plated on micropatterned fibronectin in serum-free medium, immunofluorescence labeling revealed that vinculin was localized to the patterned fibronectin foci in semilunar adhesions with concavities toward the cell center. These findings demonstrate spatial control over focal adhesion formation, and provide a foundation to investigate mechanisms by which different molecules contribute to the contacts these cells make with the extracellular environment.

2544

### Taking Cell Mechanics to the Third Dimension

P. Panorchan,[1] J. S. Lee,[1] Y. Tseng,[2] D. Wirtz[1]; [1]Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, [2]Chemical Engineering, University of Florida, Gainesville, FL

Cell function and morphology are profoundly affected by the dimensionality of the extracellular environment confining the cell. How cells mechanically sense the dimensionality of their local environment - whether plated on a 2D substrate or embedded in a 3D matrix - is mostly unknown, partly because individual cells in an extended 3D matrix are inaccessible to physical probes. Here we develop a high-resolution functional assay to measure the intracellular micromechanical response of single human umbilical vein endothelial cells (HUVECs) imbedded in a matrix and compare the response of single HUVECs plated a flat substrate coated with the same matrix. In addition to their different morphology and actin cytoskeleton organization, the cytoplasmic viscoelasticity of cells in a matrix differ sharply from that of cells flattened on a substratum. Treatment with vascular endothelial growth factor (VEGF), a promoter of angiogenic migration, induces opposite micromechanical responses in 2D and 3D. This differential mechanical response is abrogated by Y-27632, a specific inhibitor of Rho-kinase (ROCK). These results suggest that endothelial cells sense mechanically the dimensionality of their confining environment and respond to VEGF primarily through ROCK-mediated contractility.

2545

### Stressed Polystyrene Causes Increased Membrane Sensitivity of Adherent Cells to Fluid Shear Force

J. Y. Lau, R. Tchao; Pharmaceutical Sciences, Div. of Pharmacology & Toxicology, University of the Sciences in Philadelphia, Philadelphia, PA

Adherent cells are able to transduce signals from its underlying extracellular matrix (ECM) resulting in changes in various cell functions, including cell spreading and morphology. However, changes in the mechanical properties of the cell membrane due to adherence to a substratum have not been studied. Adherent NBT II cells, a rat bladder carcinoma cell line, on polystyrene (PS) discs made from pure, atactic polystyrene appear to respond to the topographical difference between the peripheral 1mm zone and the center of the disc. Cells on the peripheral zone resulted in 91.5±0.42% cell death due to instantaneous rupture of the cell membrane after application of a fluid shear force, as determined by the Live/Dead® cell assay kit (Molecular Probes). NBT II cells on the center of the disc and on the surrounding glass surface showed little cell death with a viability of 89.7±1.06% and 94.7±0.14%, respectively. Under cross-polarized light, the edge of the PS disc showed a low degree of birefringence with no birefringence at the center of the disc. Since birefringence suggests a more orderly arrangement of the PS polymer molecules, we applied various weights (1g, 5g, 10g, 20g, and 40g) to the PS disc at 100°C for 2 hours and then cooled rapidly at 4°C. We have found that increasing weights resulted in increasing degrees of birefringence. In contrast to NBT II cells on the center of the PS disc or unstressed PS, cells on the stressed PS resulted in 50.7±10.9% cell death due to membrane rupture after a fluid shear force. Interestingly, increased birefringence is also associated with increased lipophilicity, as determined by Nile Red staining. We propose that NBT II cell interaction with stressed PS results in cell sensitivity to mechanical forces.

2546

### Evaluating the Behavior of Neural Cells Near an Electrode in Rat Brain Slice Cultures

B. A. Koeneman,[1] A. Panitch,[2] J. He,[3,2] D. G. Capco[1]; [1]School of Life Sciences, Arizona State University, Tempe, AZ, [2]Harrington Department of Bioengineering, Arizona State University, Tempe, AZ, [3]Arizona Biodesign Institute, Arizona State University, Tempe, AZ

Glial scarring in the brain impedes the optimal functionality of implanted neural recording electrodes. Composition of the electrode is a leading cause of the scar formation. By utilizing a three-dimensional tissue model, a method to evaluate the biocompatibility of novel electrode materials is described. The model involves organotypic tissue culture for maintaining healthy neuronal tissue slices in the absence of the systemic response. While limitations of any in vitro model exist, brain slice culture better mimics the in vivo conditions of glia and neurons than the more traditional two-dimensional cell culture. Another important advantage of brain slice cultures is that fewer animals can be used, since several slices can be obtained per animal. Living brain slices derived from the hippocampal regions of 100 g CD rats were utilized to evaluate the biocompatibility of the BCB neural electrodes. Due to the low water uptake and inherent flexibility when formed in thin sheets, poly(benzylcyclobutene) (BCB) electrodes were developed as test materials. Cellular response to the electrodes was evaluated at 0, 7, and 14 days. At the various time points live/dead viability assays were performed as well as immunohistochemistry to determine the effects of the neural electrodes. Living cells were observed both in the vicinity of the implant, within the brain slice, as well as migrating onto the shank of the electrode. In conclusion, both neurons and glia can be successfully evaluated in a native three-dimensional state by utilizing brain slice cultures, while sacrificing fewer animals.

Supported by DARPA funding.

## Integrins (2547-2568)

2547

### Amino Acid Changes in Drosophila αPS2βPS Integrins that Affect Ligand Affinity

T. A. Bunch,[1] T. L. Helsten,[2] T. L. Kendall,[1] K. Shakalya,[3] D. Mahadevan,[3] S. J. Shattil,[2] D. L. Brower[1]; [1]Molecular and Cellular Biology, University of Arizona, Tucson, AZ, [2]Department of Medicine, University of California San Diego, La Jolla, CA, [3]Department of Medicine, Arizona Cancer Center, Tucson, AZ

We have developed a ligand-mimetic antibody Fab fragment specific for *Drosophila* αPS2βPS integrins to probe the ligand binding affinities of these invertebrate receptors. TWOW-1 was constructed by inserting a fragment of the ECM protein, Tiggrin, into the backbone of the αvβ3 ligand-mimetic antibody WOW-1. As seen for other integrins, αPS2βPS affinity for TWOW-1 is increased by mutations in the αPS2 membrane-proximal cytoplasmic GFFNR sequence, or by exposure to $Mn^{2+}$. Although $Mn^{2+}$ is sometimes assumed to promote maximal integrin activity, we find that TWOW-1 binding in $Mn^{2+}$ can be further increased by the αPS2 GFFNR > GFANA mutation. A mutation in the βPS I domain (βPS-b58; V409 > D) greatly increases integrin binding to TWOW-1, as predicted from cell spreading studies. Mutations that potentially reduce interaction of the integrin β subunit PSI and stalk domains have been shown to have activating properties. We find that complete deletion of the βPS PSI domain enhances TWOW-1 binding. Moreover, the PSI domain is dispensible for at least some other integrin functions, as these mutants display an enhanced ability to mediate cell spreading. Integrins containing the αPS2m8 (missing exon 8) splice variant show reduced binding to TWOW-1 relative to the full length αPS2C. However, the two forms display similar affinities in the presence of $Mn^{2+}$, suggesting that previously characterized differences in αPS2m8 and αPS2C ligand preference result from conformational differences, as opposed to specific contacts between ligand and exon 8-encoded residues. These studies establish a means to evaluate mechanisms and consequences of integrin affinity modulation in a tractable model genetic system.

2548

### SDF-1 and Cytochalasin D Effects on Beta2 Integrin Avidity Differ in their Functional Consequences for Cooperativity Between Beta2 Integrins and E-selectin Ligands

K. Gupta,[1] X. Wu,[1] T. Yu,[1] D. F. Kucik[1,2]; [1]Pathology, UAB, Birmingham, AL, [2]Pathology, Birmingham VA Medical Center, Birmingham, AL

$\beta_2$ integrins on leukocytes increase their adhesiveness both by affinity changes and by rearrangements (clustering). Selectin ligands on leukocytes also rearrange in response to selectin binding and as yet incompletely defined extracellular and intracellular signals. Although selectins and $\beta_2$ integrins have been thought to act sequentially, recent evidence indicates that the functions of these adhesion molecules overlap. Our recent work has shown that cooperation between E-selectin ligand and $\beta_2$ integrins is particularly important in a key phase of monocyte activation: avidity enhancement by integrin rearrangement. In the current study, we used low-dose cytochalasin D, an established method that separates avidity from affinity effects on $\beta_2$ integrins by releasing the integrins from cytoskeletal constraints on their motion (confirmed by diffusion measurements). This treatment has been shown to increase avidity and clustering without changing binding affinity. Low-dose cytochalasin D (0.3-1.0 μg/ml) increased the number of cells captured under flow on a substrate of mixed E-selectin and ICAM-1 (E+I); however it had no effect under flow conditions on adhesion to a substrate of either adhesion molecule alone. SDF-1α induces both affinity changes and rearrangement of $\beta_2$ integrins. SDF-1α had little or no effect on the number of cells captured by an E+I substrate, but transiently slowed rolling velocity; this was inhibited by pertussis toxin. The SDF-1α and cytochalasin D effects were additive, suggesting that the mechanisms of action differ at the level of the adhesion molecules. Both effects, however, required the presence of both integrin ligand and selectin in the adhesive substrate. These experiments demonstrate that SDF-1α avidity changes are less effective in promoting capture on E+I than cytochalasin D-induced clustering, but SDF-1α has a greater effect on rolling velocity.

2549

### Effects of Amino Acid Substitutions in and Around the Arginine-Glycine-Aspartic Acid (RGD) Sequence on Fertilization and Parthenogenetic Development in Mature Bovine Oocytes

B. R. Sessions, K. I. Aston, A. P. Davis, B. J. Pate, K. L. White; Animal Dairy and Veterinary Sciences and Center for Integrated Biosystems, Utah State University, Logan, UT

Integrins have been shown to be involved in the process of fertilization and many integrin-ligand interactions are mediated through the recognition of an arginine-glycine-aspartic acid (RGD) sequence. Despite the fact that the RGD domain is a principal player in determining the functional characteristics of an adhesive protein, increasing evidence has accumulated implicating the flanking amino acids adjacent to the RGD sequence determine the functional properties of the RGD-containing protein. A set of linear peptides with modified amino acid sequences in and around the RGD tri-peptide was synthesized to better understand the specificity of the RGD-receptor interaction. Mature oocytes were fertilized in vitro in the presence of RGD-containing, RGD-modified, and non-RGD peptides. Both the RGD-containing and RGD-modified peptides impaired the ability of sperm to fertilize the bovine oocyte, illustrated by a reduction in cleavage. The linear modified RGD containing peptides were also examined for their ability to induce parthenogenetic development with the objective of providing a linear RGD peptide with greater biological activity than the one (G**RGD**SPK) reported previously by this laboratory (Campbell et al. 2000). The data demonstrate the specificity of the receptor for the RGD sequence and further implicate the involvement of integrins in the process of fertilization. The data support the findings that a linear RGD peptide can block fertilization and that amino acids around the RGD sequence have an impact on the biological activity of the receptor. These data also illustrate the importance of the amino acids surrounding the RGD sequence in determining the binding and functional properties of RGD-containing peptides.

2550

### Integrin-Ligand Bonds are Catch Bonds

D. Boettiger, Q. Shi; Microbiology, University of Pennsylvania, Philadelphia, PA

The function of adhesive bonds requires that they be able to resist an applied force. When force is applied to a receptor-ligand bond, the resulting tension can alter the molecular interactions in the binding interface. If these result in no increase or a decrease in binding strength, the bond are slip bonds. If tension results in an increase in binding strength, the bonds are catch bonds. We analyzed the function of adhesive α5β1 integrin-fibronectin bonds using chemical cross-linking and the spinning disc. In Ht1080 fibroblasts spreading on a fibronectin-coated surface the α5β1-fibronectin bonds are tensioned by the action of myosin contracting the actin cytoskeleton. When this reaction was blocked with pharmacological inhibitors, the bonds were not tensioned and could not be cross-linked using DTSSP (cell impermeant homobifunctional) cross-linkers. Appling tension to the bonds with the spinning disc restored the ability of DTSSP to crosslink the α5β1-fibronectin bond. Blocking actin assembly did not prevent the formation of α5β1-fibronectin bonds but prevented both the DTSSP cross-linking and the tensioning of the bonds by the spinning disc. In the presence of low doses of cytochalasin D, increasing of the force using the spinning disc resulted in an increase in the force required for cell detachment. These results show that tension alters the α5β1-fibronectin bond and converts it to a bond that shows a higher resistance to detachment. The ability of these bonds to resist higher tension could be a property of single bonds, as suggested for selectins, or it could be a property of α5β1 as presented on the cell surface involving other binding proteins. Previous analysis have shown that α5β1-mediated signaling to induce the phosphorylation of FAK Y397 was tension dependent. The conformation changes that accompany tension would provide a mechanism to connect adhesion to cell signals.

2551

### Integrins and Syndecans Mediate Unique Cellular Responses to Nanoscale Topographic Cues

J. D. Foley,[1] T. J. Porri,[2] F. Yokoyama,[3] M. Nomizu,[3] P. F. Nealey,[2] C. J. Murphy[1]; [1]School of Veterinary Medicine, University of Wisconsin, Madison, WI, [2]Chemical and Biological Engineering, University of Wisconsin, Madison, WI, [3]Laboratory of Clinical Biochemistry, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

Objective: We have shown that nanometer length features affect a wide range of fundamental behaviors differently than do micron scale features. In addition we have found that nanometer length topographic features modulate Rho GTPase family members as well as multiple kinase signaling pathways. The mechanism(s) by which signaling pathways and behaviors are modulated remain unknown, but may involve ligation of specific cell matrix receptor families. Therefore, we used receptor-specific ligands to test the individual and cooperative roles of integrins and syndecans in these processes. Results: Self-assembled monolayers (SAMs) in which 0.1% of the triethylene glycol (EG(3)) was conjugated to specific ligands were formed on gold-coated silicon surfaces with pitches (groove width + ridge width) ranging from 400 to 4,000 nm. Primary human corneal epithelial cells (HCECs) were cultured on these surfaces for 12 hours and their spreading and alignment responses were measured. We found that an RGD-containing peptide promoted a scale-dependent shift in HCEC orientation such that cells preferentially aligned parallel to 400 nm pitch patterns, but perpendicular to 4,000 nm pitch. Surprisingly, this shift in alignment mirrors what was observed when EF-1, a non-RGD integrin peptide, was adsorbed onto 400 versus 4,000 nm pitch topographic features. This suggests that this scale-dependent shift in alignment is a general feature of integrin ligation and not sub-type specific. Co-presentation of a syndecan ligand significantly decreased the RGD-induced alignment on 400 nm pitch surfaces and promoted a roughly equal preference for parallel and perpendicular alignment on 4,000 nm pitch. Conclusions: These data suggest that integrin and syndecans promote unique cellular responses to topography, that integrins play a role in scale-dependent modulation of cellular behaviors and that this modulation can be altered by co-presentation of syndecan ligands.

2552

### Functional Overlap between Dystrophin and the α7β1 Integrin

J. E. Rooney; Pharmacology, University of Nevada, Reno, Reno, NV

Duchenne muscular dystrophy (DMD) is the most common X-linked human disease, with a prevalence of 1 in every 3,500 live male births. DMD patients and *mdx* mice (the murine model for DMD) have mutations in the dystrophin gene that result in the absence of the dystrophin protein. A second laminin binding complex in skeletal muscle is the α7β1 integrin. The absence of the α7 integrin results in congenital myopathy in mice and humans. The transgenic over-expression of the α7 integrin chain in the skeletal muscle of severely dystrophic mice has been show to partially rescue the diseased phenotype. These correlations suggest that dystrophin and the α7β1 integrin may have complementary and overlapping functional and structural roles in maintaining skeletal muscle integrity. In order to test this hypothesis, mice deficient for both dystrophin and the α7 integrin (*mdx*/$\alpha7^{-/-}$) were generated. These animals display severe signs of muscular dystrophy and die prematurely at 3-4 week of age. The muscle fibers of *mdx*/$\alpha7^{-/-}$ mice display extensive loss of membrane integrity, increased inflammatory cell infiltrate, fibrotic deposition and degeneration. These mice were also found to have increased muscle regeneration and changes in the expression patterns of laminin-2/4 and utrophin. Collectively, these results point to overlapping roles for dystrophin and the α7β1 integrin in skeletal muscle structure and function. The elucidation of these overlapping functional capacities may lead to the identification of novel therapies for muscular dystrophy through the exploitation and manipulation of cell signaling pathways or proteins shared by both complexes.

2553

### Role of Integrin αIIbβ3 in Platelet Activation

D. Westrup, T. Scholz, W. Wegert, C. M. Kirchmaier; Deutsche Klinik fur Diagnostik, Wiesbaden, Germany

During the early phase of blood clot formation platelets play a critical role (i) by forming clumps at the site of injury and (ii) by amplifying a proteolytic coagulation cascade that culminates in the activation of thrombin, cleavage of fibrinogen and formation of the fibrin network. Platelet aggregation depends on the integrin αIIbβ3 (glycoprotein IIb/IIIa; CD41/CD61). Upon activation, αIIbβ3 forms high-affinity interactions with fibrinogen, fibronectin and von Willebrand factor. Binding of fibrinogen to αIIbβ3 mediates clot formation by crosslinking platelets to each other. In addition, binding of ligands to αIIbβ3 has been shown to trigger a reorganization of the actin cytoskeleton. Here we have studied the role of αIIbβ3 for platelet-mediated amplification of the proteolytic coagulation cascade. Platelets were obtained from patients with Glanzmann's thrombasthenia, a bleeding disorder caused by mutations in αIIbβ3. Compared to controls, platelets with defects in αIIbβ3 exhibited significantly reduced induction of markers for platelet activation, including reduced surface expression of phosphatidylserine and impaired exocytosis of secretory granules. Moreover, αIIbβ3-deficient platelets failed to optimally catalyze the activation of thrombin. Taken together, these results support the conclusion that activation of αIIbβ3 is required for optimal induction of platelet-mediated fibrin formation.

2554

**Vav Guanine Nucleotide Exchange Factors and Rac are Required for Actin Polymerization during Complement-mediated Phagocytosis**

A. B. Hall,[1] M. Angelica Martinez Gakidis,[1] M. Glogauer,[2] J. Wilsbacher,[1] W. Swat,[3] J. S. Brugge[1]; [1]Cell Biology, Harvard Medical School, Boston, MA, [2]Faculty of Dentistry, University of Toronto, Toronto, ON, Canada, [3]Pathology and Immunology, Washington University School of Medicine, St. Louis, MO

Phagocytosis is an important biological process and represents a useful model for studying spatially and temporally defined events involved in actin cytoskeletal rearrangements triggered by the engagement of signaling receptors. Vav family guanine nucleotide exchange factors (GEFs) have been implicated in cell adhesion by integrin and immune response receptors through the regulation of the Rho GTPases. We are examining the role of Vav and the Rho GTPases in phagocytosis using primary bone marrow-derived murine macrophages. The genetic deletion of both Rac1 and Rac2 prevents phagocytosis mediated by integrin and Fcγ receptors (FcγR), whereas the genetic deletion of Vav1 and Vav3 or all three Vav family members prevents only integrin-mediated phagocytosis through the complement receptor $\alpha_M\beta_2$. In addition, a Rac1/2 or Vav1/3 deficiency blocks Arp2/3 recruitment and actin polymerization at the complement-induced phagosome, indicating that Vav and Rac family proteins regulate these early steps in phagocytosis. In contrast, the specific inhibition of Rho with C3 transferase prevents both complement and FcγR-mediated phagocytosis at a step distinct from actin polymerization. Moreover, a constitutively active mutant of Rac, but not Cdc42, is able to rescue actin polymerization and complement-mediated phagocytosis in Vav-deficient macrophages. These studies indicate that Rac is critical for both complement and FcγR- mediated phagocytosis and plays a broader role than originally revealed in studies of immortalized cell lines. In contrast, Vav is specifically required for complement-mediated phagocytosis, suggesting that Rac is regulated by GEFs other than Vav downstream of the FcγR.

2555

**Novel Role for Tetraspanin CD81 in Photoreceptor Outer Segment Phagocytosis by Retinal Pigment Epithelial Cells**

Y. Chang,[1,2] S. C. Finnemann[1,2]; [1]Graduate Program of Physiology, Biophysics & Systems Biology, Weill Medical College of Cornell University, New York, NY, [2]Departments of Ophthalmology, and of Cell and Developmental Biology, Dyson Vision Research Institute, Weill Medical College of Cornell University, New York, NY

Retinal pigment epithelial (RPE) cells are among the most active phagocytic mammalian cell types. Every day, each RPE cell engulfs and digests numerous outer segment fragments (OS) shed in a circadian rhythm by adjacent photoreceptor cells. αvβ5 integrin receptors of the RPE and their downstream signaling pathways are required for this rhythmic phagocytic event that is critical for vision. We hypothesize that the activity of αvβ5 integrin itself may be regulated in the RPE to synchronize phagocytosis. Tetraspanins are a family of transmembrane proteins that can regulate integrin receptor activity. Integrin-tetraspanin complexes have been shown to localize in cholesterol-rich membrane domains to initiate integrin downstream signaling. Here, we test whether the tetraspanin protein CD81 functionally interacts with αvβ5 integrin in RPE cells. CD81 is known to localize to the apical, phagocytic surface of the RPE in the eye. We used confocal microscopy to characterize RPE cells labeled live on ice with CD81 and αvβ5 antibodies. We found that CD81 partially co-localized with αvβ5 integrin at the phagocytic surface of RPE cells in culture. Optiprep gradients showed that CD81 co-fractionated with αvβ5 in low-density domains of RPE cell lysates. Furthermore, we identified CD81 in a complex with αvβ5 integrin by co-immunoprecipitation. Finally, phagocytosis assays showed that CD81 blocking antibody reduced phagocytosis of isolated photoreceptor OS by human and rat RPE cells in culture. These data suggest that apical surface CD81 plays an important role in the phagocytic function of the RPE, possibly through its interaction with αvβ5 integrin and membrane microdomains. This could provide a novel mechanism for modulating αvβ5 integrin receptor activity and thus RPE phagocytosis. We are currently studying CD81 and integrin αvβ5 complex formation and subcellular localization during active RPE phagocytosis.

2556

**β1 Integrin Regulates αvβ3 Integrin Ligand Binding Via a PKA/Calcineurin-Dependent Pathway**

A. M. Gonzalez, J. Claiborne, J. C. R. Jones; Cell and Molecular Biology, Northwestern University, The Feinberg School of Medicine, Chicago, IL

The interaction of endothelial cells with certain extracellular matrix components is regulated by a complex interplay between αvβ3 integrin and β1 containing integrins (Gonzalez et al., 2002, PNAS). To understand the mechanism underlying this interplay, we have analyzed endothelial cell interaction with the G domain of the α4 laminin which contains distinct high affinity binding sites for αvβ3 and α3β1 integrin. A β1 integrin antagonist efficiently inhibits αvβ3 integrin-mediated adhesion of endothelial cells to ligand indicating that β1 integrin-containing heterodimers function to regulate αvβ3 integrin ligand affinity via an inside-out mechanism. Clustering of β1 integrin with an antibody antagonist results in a two-fold increase in PKA activity in endothelial cells and a concomitant increase in serine phosphorylation of the β3 integrin subunit. Moreover, pharmacological inhibition of PKA rescues αvβ3 integrin-mediated endothelial cell adhesion to ligand in the presence of a β1 integrin antagonist. Since the β3 cytoplasmic tail lacks a PKA consensus site, it is unlikely that PKA is acting directly on β3 integrin. Rather, we have tested the hypothesis that PKA inhibits the calcineurin/inhibitor-1 pathway, blocking the activation of downstream protein phosphatase 1 (PP1) that, in turn, regulates the phosphorylation state of αvβ3 integrin. In support of this, endothelial cell adhesion to ligand is dramatically reduced if the cells are treated with either FK506 and cyclosporin A, inhibitors of calcineurin, or the phosphatase inhibitors okadaic acid and calyculin A, in combination with a PKA and a β1 integrin antagonist. Taken together these results indicate that PKA and the calcineurin/PP1 pathway play important roles in regulating αvβ3 integrin adhesion and signaling in endothelial cells by maintaining a balance between the phosphorylation/ dephosphorylation state of β3 integrin.

2557

**Effect of Biologically Active Peptide-Chitosan Membrane on Cell Morphology and Proliferation**

T. Watanabe,[1] M. Mochizuki,[1,2] N. Yamagata,[1] Y. Kikkawa,[1] J. Yamada,[1] M. Nomizu[1]; [1]School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan, [2]Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan

Extracellular matrix (ECM) plays a critical role for diverse biological activities including promotion of cell adhesion, migration, spreading, neurite outgrowth, angiogenesis, and wound healing. Various biologically active peptides derived from extracellular matrix components, including laminin, collagen, and fibronectin, have been identified. Previously, we conjugated biologically active peptides derived from laminin to chitosan

membranes. The peptide-chitosan membranes showed strong cell attachment activities and have been suggested to have a potential to apply for tissue engineering as basement membrane mimetic. Here, we focused on integrin-binding peptides derived from ECM components. Integrins, a family of receptors for ECM components, mediate various biological activities including cell adhesion, migration, spreading, neurite outgrowth, angiogenesis, and wound healing. Various integrin-binding peptides were conjugated onto a chitosan membrane and examined their effect on cell adhesion, morphology and proliferation using HT-1080 human fibrosarcoma cells and human foreskin fibroblasts. Most of the peptide-chitosan membranes promoted cell spreading with cell-type specific manner. The morphological appearance of the cells on the peptide-chitosan membranes was peptide-specific. The RGD sequence containing peptide (A99: AGTFALRGDNPQG and FIB1: YAVTGRGDSPAS)-chitosan membranes interacted with αvβ3 integrins and promoted neurite outgrowth with PC12 rat pheochromocytoma cells. However, most of the integrin binding peptide-conjugated chitosan membranes did not promoted neurite outgrowth activity. These results suggested that biological activities on the integrin binding peptide-chitosan membranes are integrin type-specific. These integrin binding peptide-chitosan membranes are useful to develop biomedical materials.

2558

**Molecular Basis of Integrin-Filamin A Interaction and its Competition by Talin**

Y. Lad,[1] T. Kiema,[2] P. Jiang,[3] K. L. Wegener,[3] I. D. Campbell,[3] J. Ylänne,[2,4] D. A. Calderwood[1]; [1]Department of Pharmacology and Interdepartmental Program in Vascular Biology and Transplantation, Yale University School of Medicine, New Haven, CT, [2]Biocenter Oulu and Department of Biochemistry, University of Oulu, Oulu, Finland, [3]Department of Biochemistry, University of Oxford, Oxford, United Kingdom, [4]Department of Bio and Environmental Science, University of Jyväskylä, Jyväskylä, Finland

Integrin-mediated cell migration is critical in many physiological and pathological processes such as embryonic development, leukocyte trafficking, angiogenesis and tumour metastasis. Binding of the large actin cross-linking protein filamin A to the cytoplasmic tails of integrin adhesion receptors regulates cell migration, and filamin A mutations cause a wide spectrum of diseases, including defective neuronal migration. Human filamins are composed of two 280 kDa subunits; each subunit contains an N-terminal actin-binding domain, composed of two calponin homology domains, followed by 24 tandem immunoglobulin-like domains (IgFln1-24) interrupted by flexible hinge regions between domains 15 and 16, and 23 and 24. Dimerization occurs through IgFln24. Here we map a major integrin-binding site in filamin A to a single filamin immunoglobulin-like (IgFln) domain and describe the three-dimensional structure of this domain bound to a peptide from the integrin β7 tail. NMR titration, X-ray crystallography and mutagenesis reveal that when bound to filamin, the C-terminal portion of the integrin β tail forms an extended β-strand that interacts with β-strands C and D of the IgFln domain. We show that this interaction is common to many integrins and suggest that the CD face of IgFln domains represents a general ligand-binding surface. The filamin-binding site on integrin overlaps with that of the key integrin-regulator talin, and these proteins compete for binding to integrin. Modulation of this competition, perhaps by phosphorylation, may regulate integrin function and cell migration.

2559

**Integrin α6β4 Contributions to an Invasive Phenotype in Pancreatic Carcinoma**

Z. Cruz-Monserrate, K. L. O'Connor; Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, TX

Pancreatic cancer is one of the most lethal human cancers due to an elevated incidence of tumor cell invasion and metastasis. The general mechanisms and molecules involved in this processes are not yet understood. Our study focuses on integrin α6β4, which correlates with the motile and invasive phenotype of multiple types of carcinomas. To assess the importance of integrin α6β4 in pancreatic cancer, pancreatic tumors and cell lines were screened for the presence of integrin α6β4 via immunohistochemistry, immunoblotting, and fluorescence activated cell sorting. We found that integrin α6β4 is upregulated in pancreatic tumors when compared to normal pancreas and its expression correlated with tumor progression. Integrin α6β4 cell surface expression correlated with their ability to migrate and invaded towards hepatocyte growth factor (HGF). HGF is a known mitogenic and motility factor of pancreatic carcinomas, whose receptor c-Met cooperates with integrin α6β4 to enhance cancer cell motility and invasion. When cells expressing high levels of integrin α6β4 where treated with siRNA targeting the β4 integrin subunit, we observe a reduction in the ability of cells to migrate and invade towards HGF when compare to cells treated with siRNA to a nonspecific target. Furthermore, integrin α6β4 is known to promote invasion of breast and colon cancer cells via the activation of phosphoinositide-3 kinase (PI3-K). We find that PI3-K inhibitors decreased the ability of cells that have high levels of integrin α6β4 expression on the cell surface to invade towards HGF. Furthermore, we find that the activity of the small GTPase Rac-1 is related to changes in α6β4 integrin expression. We conclude that integrin α6β4 plays an important role in the invasive phenotype of pancreatic carcinoma cells and that the PI3-K/Rac1 pathway is an important mediator of integrin α6β4-mediated invasion and migration.

2560

**Role of Beta-turn of Integrin αv-chain in the Regulation of CD44 Surface Expression, MMP2 Secretion and Migration of Human Melanoma Cells**

S. Venkatasababa, H. Wei, D. Ego-Osuala, M. A. Chellaiah; Department of BMS, University of Maryland, Baltimore, MD

The level of integrin αvβ3 and expression of its ligand osteopontin (OPN) has been directly correlated to tumorigenicity of melanoma. The M21-L is a melanoma cell line lacking αv expression and hence also the αvβ3 integrin. M21-L cell line was used for the expression of αv (M21 cells) and cytoplasmic domain deleted αv (M21-L/αv995). M21 cells were used for the expression of OPN (M21/OPN) and mutated OPN (RGA). An increase in c-Src kinase activity associated with αvβ3 was observed in M21/OPN cells as compared with M21 cells. This association was not observed in M21-L/αv995 cells. To further corroborate the binding site of c-Src in the cytoplasmic domain of αv, three peptides corresponding to the αv cytoplasmic domain were made. The β-turn peptide of αv consisting of aa sequence '995RPPQEEQERE1004' bound to several signaling such as, talin, Src, FAK, paxillin, and a few uncharacterized proteins. Either negligible or no binding of proteins with peptides corresponding to aa 986- 994 or aa 1005-1018 was observed. In a wound closure assay, M21/OPN cells exhibit an enhanced ability to migrate and this is blocked by αv and MMP2 inhibitors as well as by a blocking antibody to CD44. An increase in CD44 surface expression as well as MMP2 activity in the conditioned medium was observed in M21/OPN cells as compared with M21 cells. Basal level activity of MMP2 was observed in M21/OPN (RGA), M21-L, and M21-L/αv995 cells. Our observations suggest that the aa sequence in the β-turn of αv chain is indispensable for αv-associated

signaling complex formation and out-side-in signaling. OPN is a unique ligand for αvβ3. Its integrin activation potential increases CD44 surface expression and MMP2 activity in melanoma cells.

2561

**Integrin α6 Clipping: A Novel Mechanism to Modulate Cell Motility**

S. C. Pawar, M. C. Demetriou, R. B. Nagle, G. Bowden, A. E. Cress; Arizona Cancer Center, University of Arizona, Tucson, AZ

Integrins play a major role in cell adhesion and migration. A clipped form of the integrin α6 called integrin α6p has been detected in invasive human prostate cancer tissue and is absent in normal prostate tissue. Integrin α6p is generated by Urokinase-type plasminogen activator (uPA) mediated clippage of the integrin α6 at the cell surface. We identified the clippage site and determined if the clippage of integrin α6 to α6p altered cell migration. Using site-directed mutagenesis we identified residues R594 and R595 essential for clipping, in the "stalk" region of integrin α6. Prostate cancer cells (PC3N) were stably transfected to overexpress the clippable, wild-type form of integrin α6 (PC3N-α6-WT) or the unclippable form of integrin α6 (PC3N-α6-RR) in which the two required arginines were replaced by two alanines. Induced migration of PC3N-α6-RR cells on Laminin-5 was reduced up to 2.4 fold as compared to the PC3N-α6-WT cells. Stimulated migration on the integrin α6 ligand, Laminin-5, also induced production of integrin α6p in the PC3N-α6-WT cells and not in the PC3N-α6-RR cells. These data suggest that the cell surface clipping of the α6 integrin extracellular domain may be involved in tumor cell migration on Laminin-5.

2562

**The Integrin Alpha9beta1 Contributes to Granulopiesis by Enhancing G-CSF Signaling**

C. Chen, X. Huang, D. Sheppard; Lung Biology Center, UCSF, San Francisco, CA

The integrin α9 subunit forms a single heterodimer, α9β1. The integrin α9β1 is a receptor for a diverse group of ligands, including the extracellular matrix proteins osteopontin and tenacsin C, the cell surface immunoglobulin vascular cell adhesion molecule-1 and the lympangiogenic growth factors, VEGFC and D. Previous studies show that mice homozygous for a null mutation in the α9 subunit gene appear normal at birth but develop respiratory failure and die between 6 and 12 days of age from congenital chylothorax, suggesting a role for this integrin in lymphatic development. We now show that integrin α9-deficient mice also have a dramatic defect in neutrophil development. α9 knockout mice have decreased numbers of circulating neutrophils and decreased numbers of granulocyte precursors in bone marrow. Bone marrow cells from α9 knockout mice or human bone marrow cells blocked by anti-α9β1 antibody show a defect in differentiation to granulocytes in response to G-CSF. When stimulated with G-CSF in vitro, α9-deficient bone marrow cells or human bone marrow cells blocked with anti-α9β1 antibody demonstrated decreased STAT3 activation in response to G-CSF. When wild type or mutant integrin α9 and G-CSF receptor were co-expressed in CHO cells, STAT3 phosphorylation in response to GCSF required ligation of α9β1 and was dependent on the presence of the authentic α9 cytoplasmic domain. We conclude that integrin α9β1 is required for granulopoiesis and that integrin ligation plays a permissive role in the G-CSF signaling pathway, a process that depends on the presence of specific sequences within the α9 cytoplasmic domain.

2563

**The Mechanism of Angiogenesis Inhibition by Blocking of α1β1 Integrin**

I. Oliva, I. Staniszewska, C. Marcinkiewicz; Department of Neuroscience, Temple University, School of Medicine, Philadelphia, PA

Disintegrins are cystein-rich proteins that interact with cell surface receptors belonging to integrin family. Obtustatin is a KTS-disintegrin characterized as a potent and selective inhibitor of α1β1 integrin. Previous reports showed potential role of this collagen receptor in angiogenesis, but the mechanism which involves α1β1 integrin in vascularization process is still unknown. Endothelial cells proliferation and migration are the important phenomenon of new vessel formation and integrins are strongly involved in this process. Obtustatin appeared to be a potent inhibitor of proliferation of cultured human microvascular endothelial cells (HMVEC) induced by complete FBS, as well as by only VEGF. This inhibition was dose-dependent (0.125-4 μM) in BrdU incorporation assay and in cell number counting. The anti-proliferative effect of obtustatin is related to its pro-apoptotic effect. This disintegrin induced apoptosis in HMVEC tested in ADP/ATP ratio measurement, Annexin V assays, and Caspase 3 assay. We focused on the MAP kinase investigating signaling pathway that is induced after binding of obtustatin to α1β1 integrin. No effect was observed for p38 and JNK, but ERK 2/1 was strongly phosphorylated after exposure of HMVEC to obtustatin. Interestingly, incubation of HMVEC with obtustatin in the presence of VEGF did not change any phosporylation ratio of ERK 1/2 in comparison with effect of separate compounds. These data suggest that pro-survival factor (VEGF) and pro-apoptotic agent (obtustatin) affect the same elements of signaling pathway resulting in MAPK ERK 1/2 phosporylation. The interference between VEGF receptors and α1β1 integrin in signal transduction may identify a new paradigm helping a better understanding of cell survival/death mechanisms. Further studies will be preformed to characterize detailed "cross-talk" elements of VEGFR and α1β1 integrin.

2564

**Inhibition of Prostate Cancer Cell Migration by Bisphosphonates through Attenuation of MMP 9 Activity and CD44 Surface Expression: A Comparative Analysis with the Studies on Osteoclasts**

B. Desai, S. Venkatasababa, M. A. Chellaiah; Department of BMS, University of Maryland, Baltimore, MD

Osteopontin (OPN) has been identified to have a role in the determination of osteotropism and growth of prostate cancer cells. In osteoclasts, we have demonstrated that binding of OPN to αvβ3 initiates intracellular signals required for the surface expression of CD44. Much of what we have learned in the osteoclasts related to αvβ3 signaling is relevant to prostate cancer cells since both cell types express OPN as an autocrine motility factor. Our results identify that PC3 cells derived from bone metastasis as well as osteoclasts exhibit MMP-9 activity to a greater extent despite some MMP-2 was also observed in PC3 cells. An increase in CD44 surface expression as well as MMP 9 activity in the conditioned medium was observed in PC3 cells over expressing OPN (PC3/OPN) as compared with PC3 cells. A significant decrease in MMP-9 activity as well as CD44 surface expression below the control level (PC3) was observed in mutant OPN expressing (PC3/OPN (RGDΔRGA)) as well as in OPN-SiRNA transfected PC3 cells (PC3/OPN-/-). Bisphosphonates is an effective inhibitor of osteoclast activation and function. Bisphosphonates such as alendronate or pamindronate reduced MMP-9 activity in the conditioned medium of these cell lines. A significant inhibition of surface colocalization of MMP-9 and CD44 by bisphosphonates was observed on the cell surface in the immunostaining analysis. Bisphosphonates and

MMP-9 inhibitor significantly reduced the migration of both PC3 and PC3/OPN cells in the wound closure assay. Our findings suggest that bisphosphonates down regulates MMP- 9 secretion and CD44 surface expression in both osteoclasts and prostate cancer cells contributing to possible reduction in invasive nature of prostate cancer cells and decreased bone resorption potential of osteoclasts.

2565

**Blocking of Collagen Receptors in Melanoma and Endothelial Cells Migration**

I. Staniszewska, I. Oliva, C. Marcinkiewicz; Department of Neuroscience, Temple University, School of Medicine, Philadelphia, PA

Collagen receptors, α1β1 and α2β1 integrins are highly expressed on the surface of endothelial cells and many metastatic tumoral cells, playing important role in tumor angiogenesis and metastasis. Viperistatin and VP12 were characterized as potent and selective inhibitors of α1β1 and α2β1 integrin, respectively. Viperistatin belongs to the snake venom disintegrins, whereas VP12 is a C-lectin type protein. We tested two human melanoma cell lines that differ in expression of αvβ3 integrin. MV3 is a cell line αvβ3 negative, while HS.939T cell line αvβ3 positive. Using an adhesion assay we observed that expression of both collagen receptors is increased on MV3 cells and this cell line showed doubled potency to adhere to immobilized collagen type I and type IV. The adhesion of both melanoma cell lines to both types of collagens was inhibited by viperistatin and VP12, but with different potency. Inhibitory effect of snake venom proteins was from one to three orders of magnitude higher for HS.939T cells in comparison with MV3. Interestingly, we observed highly synergistic effect using viperistatin in the presence of VP12. In the migration assay *in vitro* through endothelial cell layer plated on the membrane with immobilized collagen IV, viperistatin have no significant effect, whereas VP12 showed essential inhibitory effect. However, viperistatin was very potent inhibitor of transmigration of HS.939T cells in this assay, and VP12 had moderated effect. These data suggest that the major pro-migratory collagen receptor on MV3 cells is α2β1 integrin, while on HS.939T cells α1β1 integrin. VP12 also potently inhibited the dermal microvascular endothelial cell radial migration in collagen gel. Our results suggest that inhibitors of collagen receptors may have therapeutic implication in angiogenesis and metastasis of melanoma.

2566

**SWAP-70 Regulates LFA-1 Mediated Integrin Signaling Leading to Mast Cell Adhesion**

R. Sivalenka,[1] R. Jessberger[2]; [1]Dept. of Gene and Cell Medicine, Mount Sinai School of Medicine, New York, NY, [2]Institute of Physiological Chemistry Medical School, MTZ, Dresden University of Technology, Dresden, Germany

SWAP-70 regulates rearrangements of the F-actin cytoskeleton in specific cell types, e.g. in bone marrow derived murine mast cells (BMMC) through signaling from their two major receptors - FcεR1 and c-kit. Recently we found that SWAP-70 negatively regulates c-kit induced homotypic adhesion of BMMC mediated by integrin $\alpha_L\beta_2$ (LFA-1). On the contrary SWAP-70 is required for efficient adhesion to extracellular matrix proteins like fibronectin and enhances c-kit mediated adhesion to fibronectin. Thus, SWAP-70 appears to play a role in inside-out signaling to integrins. Besides its role in adhesion of BMMC, SWAP-70 is also required for migration of mast cells towards SCF (c-kit ligand). $\alpha_L\beta_2$-ICAM-1 interactions have been implicated in mast cell interactions with B and T lymphocytes, contributing to their activation, and the function of SWAP-70 in such heterotypic interactions is being investigated. Besides, the present study aims at investigating SWAP-70's role in signaling to $\alpha_L\beta_2$ and in the associated cytoskeletal dynamics. SWAP-$70^{-/-}$ BMMC, which express integrin levels similar to wildtype, show enhanced adhesion to ICAM-1. This adhesion is further increased upon c-kit stimulation. Tyrosine phosphorylation of SWAP-70 is induced upon LFA-1 stimulation, and upon c-kit stimulation, SWAP-70 co-immunoprecipitates with $\alpha_L\beta_2$. We suggest that SWAP-70 acts in regulating interactions between integrins and the cytoskeleton.

2567

**Reconstruction and Deconstruction of Integrin αIIbβ3 Activation in Heterologous Cells**

J. Han,[1] D. A. Calderwood,[2] W. Puzon-McLaughlin,[1] M. H. Ginsberg[1]; [1]Department of Medicine, The University of California, San Diego, La Jolla, CA, [2]Department of Pharmacology, Yale University, New Haven, CT

The increase in integrins' affinity for extracellular ligands ("activation") in response to agonists is a form of inside-out signal transduction that controls cell migration, assembly of the extracellular matrix, and cell adhesion. We have now engineered heterologous cells to reconstruct a signaling pathway that mediates agonist-induced activation of platelet integrin αIIbβ3. Talin binding to the integrin β cytoplasmic domain is a final step in integrin activation and platelets are rich in talin 1. Activation of protein kinase C (PKC) leads to αIIbβ3 activation in platelets, but not in heterologous cells, such as CHO cells. When talin 1 expression was increased ~ 10 fold in αIIbβ3-bearing CHO cells, stimulation with phorbol esters lead to PKC-dependent activation of αIIbβ3. A number of PKC-mediated talin phosphorylation sites were identified using mass spectroscopy; however, mutation of those sites to Ala did not block PKC-mediated activation. In addition, transfection of CHO cells with RapGAP (Rap GTPase Activating Protein) completely blocked PKC-induced αIIbβ3 activation, whereas PKC inhibitors fail to block activated RapV12-induced αIIbβ3 activation, indicating that Rap1 is downstream of PKC in αIIbβ3 activation. In sharp contrast, expression of RapGAP failed to block activation of αIIbβ3 induced by expression of an integrin binding fragment of talin (F23); however, a full length talin point mutation which blocks binding to integrin β3, failed to support αIIbβ3 activation by either PKC or RapV12. Finally, in a talin-deficient cell line, integrin activation is rescued by talin but not by RapV12, any of several Rap-specific effectors, or any combination of RapV12 and its effectors. These data establish that talin is downstream of Rap1 in integrin activation. Thus, this first complete reconstruction of a cellular signaling pathway for agonist-induced integrin activation orders the pathway: Agonist→PKC→Rap→Talin→Integrin.

2568

**Placental Insufficiency in α7 Integrin Null Mice**

J. V. Welser, N. L. Flintoff-Dye, D. J. Burkin; Pharmacology, University of Nevada, Reno, Reno, NV

The α7β1 integrin is a heterodimeric laminin receptor expressed in vascular smooth muscle. Loss of the α7 integrin chain results in partial embryonic lethality. We have previously shown that α7 null embryos exhibit cerebral vascular hemorrhaging which may play a role in the partial embryonic lethality. Placental defects in α7 null embryos may also contribute to the partial embryonic lethality observed in α7 integrin knockout mice. To determine if these vascular defects extend to the placental labyrinth, placentas from ED9.5 and 13.5 wild-type and α7 knockout embryos were analyzed. Histological analysis of the placentas from hemorrhaging α7 null embryos showed structural defects, including a reduction in the

spongiotrophoblast layer, as well as infiltration into the decidua layer. To determine whether the loss of the α7 integrin affects placental vascular integrity, vascular smooth muscle cells were analyzed using placentas from ED13.5 wild-type, non-hemorrhaging α7 null, and hemorrhaging α7 null embryos. A 1.5 fold increase in the number of vascular smooth muscle cells in non-hemorrhaging α7 null placentas compared to wild-type placentas was observed. In contrast, a 1.5 fold decrease in the number of vascular smooth muscle cells in hemorrhaging α7 null placentas compared to wild-type placentas was found. Altered vascular integrity in non-hemorrhaging α7 null and hemorrhaging α7 null placentas may contribute to the observed partial embryonic lethality. Our results confirm a role for the α7β1 integrin in placental development and show for the first time that loss of the α7 integrin results in placental insufficiency.

## Metalloproteases (2569-2583)

2569

### Wild-Type and Spontaneously Active Matrix Metalloproteinase (MMP)-1 Mutants Induce Vascular Regression in Vitro and in Vivo

W. B. Saunders, G. E. Davis; Medical Pathology and Laboratory Medicine, Texas A&M University System Health Science Center, College Station, TX

Previous work has shown endothelial cells (ECs) regulate regression of capillary tubes in vitro via activation of interstitial collagenase (MMP-1) by multiple serine proteases and stromelysin-2 (MMP-10). Herein, we investigate the role of human MMP-1 as a downstream mediator of vascular regression in vitro and in vivo. Site-directed mutagenesis was utilized to induce point mutations within the propeptide domain of MMP-1, resulting in four spontaneously active MMP-1 mutants: MMP-1 Y18A, L19A, C73S, and V75G. Using a previously described, three-dimensional (3D) regression assay, ECs were induced to express MMP-1 mutants via adenoviral delivery. ECs expressing MMP-1 mutants underwent capillary tube regression and collagen gel contraction in the absence of serine proteases, while ECs expressing GFP control or wild type MMP-1 underwent normal EC vacuole/lumen formation and did not regress. Importantly, the time to tube regression and collagen gel contraction corresponded with the degree of MMP-1 activation visible on Western blot as well as the collagenase activity in conditioned media. This serine-protease independent regression response was inhibited by the MMP inhibitors TIMP-1, α2Macroglobulin, and GM6001, and not by the serine protease inhibitors aprotinin or PAI-1. To evaluate the role of MMP-1 as a vascular regression factor in vivo, fibroblasts expressing wild type or mutant MMP-1 were utilized to either inhibit formation or induce regression of VEGF induced angiogenic fields using the CAM model of angiogenesis. In both assays, wild-type or mutant MMP-1 induced regression of early angiogenic responses. This regression response was enhanced by the co-delivery of MMP-10, consistent with previous work. Collectively, these data further support the role of human MMP-1 as a vascular regression factor, and demonstrate that spontaneously active MMP-1 mutants may provide use therapeutically in the treatment of disorders of increased vascularity.

2570

### Characterization of Matrix Metalloproteinase-1 Interaction with Human Type III Collagen

K. E. Williams, R. Chang, P. Hillas, C. Luehrs, F. Buschman, J. Polarek, D. Olsen; FibroGen, So San Francisco, CA

Matrix metalloproteinases (MMPs) are essential for normal collagen turnover, recovery from fibrosis, and vascular permeability. In this study we examine the biochemical interaction of MMP-1 and type III collagen through the use of a panel of recombinant human type III collagen mutants, including a single amino-acid mutation in the cleavage site (I785P). Pichia pastoris strains expressing the type III collagen alleles and human prolyl-4-hydroxylase were constructed, screened, and fermented prior to collagen purification from pepsin-digested cell lysates by repeated salt precipitation. The melting temperatures, hydroxyproline content, and CD spectra of FG-5013 (wild-type recombinant type III collagen) and FG-5015 (I785P) are comparable. Transmission electron microscopy analysis of fibrils formed with FG-5015 revealed the striated cross-banded structure typical of collagen fibrils, demonstrating that this mutation does not interfere with fibril formation. Biacore analysis revealed that MMP-1 binds to FG-5013 and FG-5015 with comparable affinity. However, the specific activity of MMP-1 for FG-5015 was 36-fold less than that for FG-5013. FG-5015 was also significantly less susceptible to proteolysis by MMP-8 and trypsin than FG-5013. N-terminal sequence analysis revealed that MMP-1 digestion of FG-5015 occurs at three sites: G781-I782, P785-T786, and G787-A788. These sites are in the vicinity of the wild-type cleavage site, suggesting that in the absence of the wild-type isoleucine, the relaxed tertiary structure of this region may contribute to the recognition of the new MMP-1 sites cleaved in FG-5015. This conclusion is further supported by the observation that an allele with increased hydroxyproline content in this vicinity shows no detectable cleavage. As collagen used in biomedical applications is susceptible to MMP proteolysis and ultimately degrades, FG-5015 may be used as a biomaterial with potentially enhanced *in vivo* persistence.

2571

### Histamine Regulates β-catenin Localization and MMP-2 expression in Endothelial Cells

J. L. Doyle, T. L. Haas; Kinesiology and Health Sciences, York University, Toronto, ON, Canada

β-catenin is a junctional protein that, together with vascular endothelial cadherin, maintains the integrity of endothelial cell-cell junctions. β-catenin is also able to translocate to the nucleus, where it acts as a transcriptional co-activator. During angiogenesis, endothelial cell junctions are reorganized, signaling the cell to proliferate and migrate. Proteolysis of the basement membrane and interstitial matrix, via matrix metalloproteinases (MMPs), is the next crucial step in the angiogenic process. Matrix metalloproteinase (MMP)-2 and membrane type (MT)-1 MMP are two endothelial cell MMPs previously linked to angiogenesis. Permeability agents such as histamine have been shown to be pro-angiogenic and cause reorganization of junctional structures. We hypothesize that 1) histamine is able to increase the nuclear level of β-catenin and 2) histamine increases expression levels of the MMPs through a β-catenin -dependent mechanism. Rat microvascular endothelial cells were stimulated with 100 μM histamine for 24 hours, which caused increased nuclear levels of β-catenin versus control as assessed by western blotting of nuclear extracts (2 fold increase vs. control, n=2). Both MMP-2 total protein and mRNA levels also are increased with histamine treatment (1.7 ± 0.18, $p < 0.05$, n=3 and 1.4 ± 0.66, n=2 respectively ). These results indicate that the nuclear translocation of β-catenin occurs in response to permeability agents such as histamine and that histamine increases both total protein and mRNA levels of MMP-2. These results also indicate that permeability agents have a role in endothelial junction disruption leading to nuclear translocation of β-catenin and that this may be a mechanism through which MMP-2 expression is regulated. Funding by CIHR.

2572

**Etoposide Disrupts MMP-2 Translation in Bone Marrow Stromal Cells**

S. D. Clutter,[1] L. F. Gibson[2,3]; [1]Microbiology, Immunology, and Cell Biology, West Virginia University, Morgantown, WV, [2]Pediatrics, West Virginia University, Morgantown, WV, [3]The Blood and Marrow Transplant and Hematologic Malignancy Program, Mary Babb Randolph Cancer Center, Morgantown, WV

We have previously reported that stromal cell MMP-2 is necessary for optimal support of pro-B cell chemotaxis. Following high dose exposure to the topoisomerase II inhibitor, etoposide, stromal cells display reduced MMP-2 protein expression that is not correlated with diminished mRNA, increased intracellular accumulation, or altered stability of MMP-2 protein. The MMP-2 gene contains a 5' polypyrimidine tract (5'TOP) and therefore has the potential to be translationally regulated by mTOR. To determine the mechanism by which MMP-2 protein expression is diminished in etoposide treated stromal cells we evaluated the expression or activity of proteins known to be components of the mTOR pathway including Akt, 4EBP-1, and $p70^{s6k}$. mTOR protein is phosphorylated by Akt and also binds PP2A, inhibiting its phosphatase activity. Dephosphorylation of mTOR frees PP2A, which then rapidly dephosphorylates translational regulatory proteins 4EBP-1 and $p70^{s6k}$. Phosphorylated $p70^{s6k}$ and 4EBP-1 both stimulate translation of genes containing 5'TOPs. To determine if etoposide inhibits translation of MMP-2 by disrupting this specific pathway, we evaluated phosphorylation of the translational regulatory proteins 4EBP-1 and $p70^{s6K}$ by western blot and activity of PP2A and Akt by specific activity assays. Stromal cells exposed to etoposide display decreased Akt activity and phosphorylation of 4EBP-1 and $p70^{s6K}$, and increased PP2A activity. These observations are consistent with regulation of stromal cell MMP-2 translation through the mTOR pathway.

2573

**Invasion-associated MMP-2 and MMP-9 are Up-regulated Intracellularly with Apoptosis Linked to Melanoma Cell Detachment**

A. M. Mendes, M. Strasberg Rieber, M. Rieber; Tumor Cell Biology Laboratory, Center for Microbiology & Cell Biology, Instituto Venezolano de Investigaciones Cientificas, IVIC, Caracas, Venezuela

OBJECTIVE.-Matrix metalloproteinases, like MMP-2 and MMP-9 gelatinases, show multiple functions as extracellular / cell-surface enzymes , and are recognized for their matrix-degrading ability and involvement in cell motility. Given that adherent cells decrease attachment when migrating and also detach from their substratum during apoptosis , we now investigated whether extracellular-matrix -bound gelatinases and intracellular MMP-2 and MMP-9, are modified with progression of death-inducing stimuli. METHODS.- Apoptosis was evaluated by Annexin V binding and DNA fragmentation studies. Gelatinase activity was evaluated by zymography . MMP-2 expression and PARP fragmentation were determined by immune blotting . RESULTS.-This report shows that melanoma cells undergoing death in response to 2-acetyl furanonaphtoquinone (FNQ) , increase cytosolic expression of pro- MMP-2 and intracellular activation of particulate MMP-9. These changes were associated with early activation of a substrate- attached 40 kDa gelatinase reciprocal with changes in extracellular matrix-bound activated MMP-2 . A subsequent activation of secreted MMP-9 and induction of apoptosis-associated fragmentation of poly ADP-Ribose polymerase (PARP) correlated with cell detachment . CONCLUSIONS.- Our data suggests that intracellularly activated gelatinases may cleave survival-associated substrates other than gelatin that share the **Gly-Leu/Iso-Pro** like collagen-binding acetylcholinesterase , thereby linking them to apoptosis associated with cell detachment.

2574

**Type IV Collagen Induces Matrix Metalloproteinase -9 Expressions in MCF7 Breast Cancer Cells**

P. Cortés Reynosa, T. Robledo, E. Perez Salazar; Bilogía celular, CINVESTAV, Mexico D.F., Mexico

**Background.** In breast cancer, cell migration/invasion is an important factor in the formation of solid tumours and necessary for spreading to other organs. In order to migrate, cells have to break extracellular matrix (ECM), including basement membrane (BM). ECM is a complex network of interacting molecules such as collagens and it regulates vital cell processes, including migration, growth, and differentiation. **Aims.** Our aim was to explore whether type IV collagen (CIV) induces metalloproteinase-9 (MMP-9) expression/activation and the role of Src kinase family members and epidermal growth factor receptor (EGFR) activities. **Methods.** Cells were stimulated with CIV and lysed. Cell lysates were analyzed by Western-blot using phosphospecific antibodies. EMSA's were performed using nuclear extracts and a probe representing a canonical NF-κB binding site. MMP-9 activity was determined by zymography using the supernants of MCF7 cells treated with CIV. **Results.** CIV induced FAK activation, phosphorylation of Src at Tyr-418, paxillin tyrosine phosphorylation, phosphorylation of MEK at Ser-217/Ser-221, formation of FAK-Src complex and NF-κB activation. In addition, CIV induced the expression and activation of MMP-9. Treatment with the Src kinase inhibitor PP-2 inhibited MMP-9 activity. In contrast, treatment with EGFR inhibitor AG-1478 did not inhibit MMP-9 activation.**Conclusions.** These findings demonstrate that CIV induces activation of diverse intracellular signaling molecules, including FAK, Src, MEK, the transcription factor NF-κB and the expression and activation of MMP-9. We suggest that these signaling molecules are modulating the expression of MMP-9, and then they are playing an important role in the migration/invasion process.

2575

**Inhibition of Cell Spreading and MMP-9 Expression by the IFN-induced Large GTPase, mGBP-2, Does Not Require GTPase Activity**

S. Balasubramanian, D. J. Vestal; Biological Sciences, University of Toledo, Toledo, OH

Among the many consequences of exposure to interferons (IFNs) are changes in how cells interact with their extracellular environment. IFNs do this, primarily, by modulating the expression of a wide variety of gene products. One family of gene products that contains members that are down-regulated by IFNs is the matrix metalloproteinase family, in particular MMP-1, MMP-2, and MMP-9. We demonstrate that IFN-γ treatment of NIH 3T3 cells retards their ability to spread on fibronectin. This inhibition is accompanied by reduced expression of MMP-9. Ectopic expression of the IFN-induced GTPase, mGBP-2, is sufficient to mimic this inhibition of cell spreading. Forced expression of mGBP-2 is also sufficient to down-regulate MMP-9 expression in these cells. Restoration of MMP-9 by ectopic expression is able to rescue the defect in cell spreading. To determine which region(s) of mGBP-2 was required to inhibit cell spreading, a variety of point mutations and truncations were generated for mGBP-2. The carboxy terminal region, containing α-helices 6 through 13 but lacking the amino terminal GTP binding domain, was sufficient to inhibit cell spreading. While GTPase activity was not required, the addition of the geranylgeranyl isoprenoid to the carboxy terminus was required to inhibit

cell spreading. A single amino acid change in the CaaX motif of mGBP-2 that prevents lipid addition abolished inhibition of cell spreading. Taken together we have demonstrated a mechanism for IFN-γ-mediated changes in cell interaction with fibronectin that begins with the transcriptional induction of mGBP-2 and proceeds through the mGBP-2-mediated inhibition of MMP-9 expression. Work is currently underway to identify the mechanism(s) by which mGBP-2 inhibits MMP-9 expression.

2576

**Synatxin 6, Vamp4, and Vti1b Complex Regulates MMP-9 Secretion during the Invasion of Breast Cancer Cells**

J. Jing,[1] A. A. Peden,[2] R. Prekeris[1]; [1]Cellular and Developmental Biology, UCHSC, Aurora, CO, [2]Wellcome Trust/MRC Building, Cambridge Institute for Medical Research, Cambridge, United Kingdom

Matrix metalloproteinases (MMPs) are family of endopeptidases that capable of cleaving extracellular matrix (ECM) molecules. The degradation of ECM plays an important role in regulation of cell motility and metastasis. MMP-9 is the members of gelatinase sub-family and mainly hydrolyze the components of basement membrane, such as gelatin and collagen type IV. MMP-9 is secreted by tumor, immune, and stroma cells, and is known for its role in tumor growth and metastasis. However, while the transcriptional regulation and activation of MMP-2 and MMP-9 are well understood, we know little about the mechanisms of their secretion. Several protein families , including SNAREs, have emerged as key regulators of protein secretion. SNARE proteins are divided in two subfamilies, VAMPs (also known as R-SNARE) and syntaxins (also known as Q-SNARE). The formation of stable core protein complex between VAMP and syntaxins was shown to be required for membrane fusion and specificity of protein secretion. Here we use siRNA-based screen to identify the SNARE proteins that mediate MMP-9 secretion. From all SNAREs tested only syntaxin 6 and VAMP4 inhibited MMP-9 secretion from MDA-MB-231 breast cancer cells. Using immunoprecipitation approaches we have shown that in MDA-MB-231 cells syntaxin 6 and VAMP4 form a protein complex, which includes Vti1B, another member of SNARE protein family. We also used zymopraphy in situ to show that syntaxin 6, VAMP4, and Vti1B complex is required for MMP-9 dependent gelatin degradation. Finally, we demonstrate that syntaxin 6 or VAMP4 down-regulation inhibits MDA-MB-231 cell invasion, while having minimal effect on cell motility. Thus, we propose that VAMP4, synatxin 6 and Vti1B sore complex is required for the fusion of MMP-9 transport vesicles with plasma membrane and subsequent secretion of MMP-9 to exrtracellula space.

2577

**MMP-3-induces Epithelial-mesenchymal Transition by Stimulating the Expression of the Splice Variant Rac1b**

D. C. Radisky,[1] D. D. Levy,[1] L. E. Littlepage,[2] H. Liu,[1] C. M. Nelson,[1] J. E. Fata,[1] D. Leake,[3] E. L. Godden,[3] D. G. Albertson,[2] M. Nieto,[4] Z. Werb,[2] M. J. Bissell[1]; [1]Lawrence Berkeley National Laboratory, Berkeley, CA, [2]University of California, San Francisco, San Francisco, CA, [3]Dharmacon, Inc., Lafayette, CO, [4]Instituto de Neurociencias de Alicante, San Juan de Alicante, Spain

We have previously investigated stromelysin-1/matrix metalloproteinase-3 (MMP-3), a stromal enzyme upregulated in many breast tumors, and found that MMP-3 can cause epithelial-mesenchymal transition (EMT) and malignant transformation in cultured cells. We have now identified the molecular pathways by which MMP-3 exerts these effects. We show that exposure of mouse mammary epithelial cells to MMP-3 leads to induction of a highly activated splice isoform of Rac1 known as Rac1b, and that expression of this protein stimulates increased mitochondrial production of reactive oxygen species (ROS). We find that the Rac1b-induced ROS are necessary and sufficient to stimulate expression of the transcription factor Snail and to induce EMT. Analysis of response to TGFbeta in this model system reveals that the EMT programs triggered by MMP-3 and TGFbeta are fundamentally and functionally distinct. These findings identify a novel pathway in which a component of the breast tumor microenvironment alters cellular structure in culture and tissue structure in vivo, leading to abberant cellular motility and malignant transformation.

2578

**The Matrix Metalloproteinase, MMP-3, Mediates Rapid Membrane Blebbing in Human Breast Epithelial Cells**

J. E. Fata,[1] Z. Werb,[2] M. J. Bissell[1]; [1]Life Sciences Division, Lawrence Berkeley National Lab, Berkeley, CA, [2]Dept. of Anatomy, University of California, San Francisco, San Francisco, CA

Cell contraction and membrane blebbing are evolutionarily conserved events that occur during the execution phase of apoptosis. Several members of the TNF-ligand superfamily, which are associated with the promotion of a number of pathological processes, including inflammation and cancer are also capable of inducing membrane blebbing in some cell types. The majority of these ligands are transmembrane bound but can be shed from the cell surface through proteolytic processing where soluble ligands can act as antagonists, as in the case of FAS ligand, or agonists, as seen with TNF-alpha. Here we provide evidence that the matrix metalloproteinase, MMP-3/stromelysin-1 induces rapid membrane blebbing in serum starved or cyclohexamide-treated MCF10A human breast epithelial cells. MMP-3-mediated membrane blebbing is associated with reorganization of the actin cytoskeleton, upregulation of both p53 and p38 MAP kinase activity, and loss of cell surface E-cadherin. A broad-spectrum MMP inhibitor completely abolishes these reactions. To understand the signalling cascade initiated by MMP-3, we asked whether factors down-stream of TNF-superfamily signalling were involved. We show that inhibitors against JNK and caspase-3, and RNAi reduction of MKK7, a known activator of JNK inhibit membrane blebbing. Moreover, stable expression of a dominant negative FADD (dnFADD), a downstream effector of several TNF superfamily ligands, renders MCF10A cells resistant to membrane blebbing. Together these findings indicate that MMP-3 induces cell membrane blebbing through a TNF-superfamily signalling pathway and provides an impetus to further explore this protease in inflammation and cancer. J.E.F. (jefata@lbl.gov) is supported by the Dept. of Defence BCRP (DAMD17-03-1-0486). This work was also partially supported by the Dept. of Energy to M.J.B. (DE AC03 76SF00098).

2579

**Overexpression of MMP-16 in *Xenopus* Causes Defects in Morphogenetic Movements During Gastrulation**

L. A. Walsh, S. Damjanovski; Biology, University of Western Ontario, London, ON, Canada

Matrix metalloproteinases (MMPs) are a family of highly homologous endopeptidases that cleave and remodel the extracellular matrix (ECM). Degradation of the ECM and the basement membrane is a critical component of tumor invasion and metastasis. Recently, there has been compelling evidence suggesting MMP-2 specifically can be correlated with the metastatic potential of many tumors. Once activated by other

proteases such as MMP-16, MMP-2 cleaves a broad spectrum of ECM constituents. Little is known however, about how these enzymes function during development when ECM remodeling is high. The overexpression of MMP-16 and the *in vivo* activation of MMP-2 were examined during *Xenopus* laevis development. Embryonic stage 1 embryos microinjected with MMP-16 mRNA show dose-dependant defects in blastopore closure during gastrulation. Phenotypic effects at stage 25 range from a shortened axis to severe defects in morphology. Histological sections of stage 25 embryos revealed the presence of all major tissue types. Somites however, appeared misaligned. This in part may contribute to the observed axis defects. Overexpression of MMP-16 did not affect marker gene expression in notochord, neural, endodermal and muscle tissues, suggesting phenotypes were not due to changes in cell fate specification but rather changes in morphogenic movements during gastrulation. Zymography confirmed the overexpression of MMP-16 mRNA resulted in increased gelatinase activity which suggests increased activation of MMP-2. Here we show that activation of MMP-2 can result in phenotype defects when upregulated. We also show that although the balance of MMP activity may not directly affect cell fate specification, it is still critical to ensure proper morphogenetic movements in *Xenopus* development.

2580

**Microenvironmental Regulators of Metastasis: Role of Matrix- and Mechanotransduction in Control of MT1-MMP Expression**

M. Barbolina,[1] E. Ariztia,[2] M. S. Stack[1]; [1]Cell and Molecular Biology, Northwestern University, Chicago, IL, [2]Obstetrics and Gynecology, New York University Medical Center, New York, NY

Epithelial ovarian carcinoma is the leading cause of death from gynecologic malignancy. Due to difficulties diagnosing ovarian cancer in early stages, most patients present when metastasis had already occurred. Thus, it is important to understand mechanisms of invasion and develop strategies to prevent tumor cells from spreading. The metastatic phenotype is characterized by disruption of cell-cell contacts and loss of extracellular matrix (ECM) constraints due to upregulation of ECM-degrading matrix metalloproteinases (MMP) and alterations in matrix- and mechano-transduction. We previously showed that metastatic ovarian cancer cells interact with interstitial collagens via α2β1- and α3β1-integrins, resulting in increased expression of membrane type 1-MMP (MT1-MMP). The goal of our research was to establish the role of epigenetic factors, particularly matrix status, in the transcriptional regulation MT1-MMP. We used real-time RT-PCR, western blotting and zymography to monitor changes in MT1-MMP and MMP2 levels in ovarian cancer cell lines cultured a) on 2D vs 3D collagen I; b) in a synthetic 3D Hydrogel (BD PuraMatrix); c) under conditions of static strain. Our results indicate an upregulation of MT1-MMP protein and activity following 3D collagen culture or static strain. Corresponding to the increased protein levels, an 8-fold increase in MT1-MMP mRNA was detected following 4 h of collagen culture using RT-RT-PCR. Upregulation of the transcription factors Egr-1 and -2 (20- and 3-fold, respectively) was observed at 2 h of collagen culture, suggesting that these factors participate in transcriptional control of MT1-MMP. These data implicate signal transduction through collagen binding integrins in control of MT1-MMP expression. As we have previously demonstrated that MT1-MMP co-localizes with α2β1 and α3β1 integrins at the cell-matrix interface, these results provide additional data in support of the hypothesis that matrix status influences matrix degrading potential.

2581

**Transforming Growth Factor-β Induces CD44 Cleavage that Promotes Breast Tumor Cell Migration by Up-regulation of MT1-MMP**

Y. Guo,[1] C. Su,[2] H. Wang[1]; [1]Department of Anatomy, Yang-Ming University, Taipei, Taiwan Republic of China, [2]Department of Surgery, Veterans General Hospital, Taipei, Taiwan Republic of China

Transforming growth factor-β (TGF-β), a multifunctional cytokine, is involved in cell proliferation, differentiation, migration, and pathological processes. CD44, a transmembrane receptor for hyaluronic acid (HA), is implicated in various adhesion-dependent cellular processes including cell migration, tumor cell metastasis and invasion. Recent studies have shown that CD44 expressed in cancer cells can be proteolytically cleaved at the ectodomain by the membrane type I matrix metalloproteinase (MT1-MMP), and that CD44 cleavage plays a critical role in cancer cell migration. Here, we demonstrated that TGF-β increases smad2/smad3 phosphorylation and MT1-MMP production in breast tumor cells. Treatment with cycloheximide, an inhibitor of translation, significantly reduced MT1-MMP production. These results suggested that TGF-β has the ability to induce MT1-MMP expression, and this pathway is through phosphorylated smad2/smad3 entering the nucleus to regulate gene transcription of MT1-MMP. Moreover, the induction of MT1-MMP expression by TGF-β is blocked by the specific extracellular regulated kinase-1/2 (ERK1/2) inhibitor PD98059 and by specific phosphoinositide 3-OH kinase (PI3K) inhibitor LY294002. We also found that treatment with SP600125, an inhibitor for c-Jun NH2-terminal kinase (JNK), resulted in a significant inhibition of MT1-MMP production. In contrast, the p38 MAP kinase inhibitor, SB203580, had no significant effect on MT1-MMP production. These data indicated that PI3K, ERK1/2, and JNK are also involved in TGF-β-induced MT1-MMP expression. Furthermore, TGF-β induced MT1-MMP is also able to cleave CD44 and this might promote tumor cell migration resulting in enhanced metastasis.

2582

**A Role of Transmembrane Domain of Tumor Necrosis Factor-α Converting Enzyme (TACE) in Ectodomain Shedding**

X. Li, H. Fan; Physiology, UMDNJ-RWJMS, Piscataway, NJ

TACE is a membrane-anchored metalloprotease that is essential for development and disease. TACE catalyzes regulated release of soluble growth factors and cytokines from their transmembrane precursors. It also cleaves growth factor/cytokine receptors. The primary sequence of TACE consists of an amino-terminal prodomain, a zinc metalloprotease domain, a cysteine-rich/disintegrin domain (CRD), a transmembrane domain (TM) and a cytoplasmic domain. The prodomain inhibits the protease activity by interacting with the enzyme's catalytic center, and is removed in the secretion pathway. We are interested in possible regulatory activities in other noncatalytic domains of TACE. Using transmembrane transforming growth factor-α (TGF-α) as a model substrate, we have previously shown that the cytoplasmic domain is dispensable for TGF-α release from the cell surface, even though the cytoplasmic domain undergoes regulated phosphorylation. In contrast, a point mutation (C600Y) in CRD resulted in complete inactivation of enzyme activity, suggesting an interaction between CRD and the metalloprotease domain. The objective of this current study is to evaluate the role of the TACE TM in ectodomain cleavage. We have substituted the TACE TM with the TMs of prolactin receptor and platelet-derived growth factor receptor. Transfection experiments showed that the chimeric TACE constructs, similar to wild type TACE, were processed to mature form located at the cell surface. However, the chimeras are severely defective in TGF-α cleavage. These results suggest that the TACE TM plays a regulatory role in ectodomain cleavage.

2583

### Comparative Localisation of Angiotensin-Converting Enzymes in Polarised Kidney Cells

F. J. Warner,[1] D. W. Lambert,[2] R. A. Lew,[1] A. I. Smith,[1] N. M. Hooper,[2] A. J. Turner[2]; [1]Biochemistry and Molecular Biology, Monash University, Clayton, Australia, [2]Biochemistry and Microbiology, University of Leeds, Leeds, United Kingdom

Angiotensin converting enzyme (ACE) is a zinc metallopeptidase, which functions as a key regulator of the renin-angiotensin system. ACE2, a homologue of ACE, is an 805 amino acid, type I integral membrane protein, abundant in human heart and kidney. Whilst ACE2 shares ~41% sequence identity to somatic ACE[1] and has the same membrane topology, it has a different catalytic action and different substrates to ACE. The cellular localisation and targeting of ACE2 has not been investigated. ACE2 and ACE were stably transfected into CHO cells and the polarised Madin-Darby canine kidney type II (MDCKII) cell and studied in parallel. In CHO cells, ACE2 and ACE were localised to the cell surface by indirect immunofluorescence. Both proteins expressed in CHO cells were soluble in Triton-X-100 at 4C when separated by sucrose density gradient centrifugation and were not associated with lipid rafts found in detergent-insoluble glycolipid-enriched fractions of cell membrane. In polarised MDCKII cells under steady-state conditions, ACE2 was observed predominantly at the apical surface by cell-surface biotinylation, and immunofluorescence studies. Comparatively, ACE was present on both the apical (45%) and basolateral membranes (55%). Soluble forms of ACE2 and ACE were detected in the medium taken from MDCKII cells by enzyme assays, suggesting ACE2, like ACE, is shed from the cell surface. ACE2 is shed apically, whereas ACE is shed from both surfaces. The level of ACE shed from the apical surface was 2.5-fold higher than that from the basolateral surface after 6 h. This study has demonstrated ACE2 and ACE are cell surface proteins that have distinct localisation patterns, that may determine their roles as ectoenzymes at the cell surfaces of polarised kidney epithelium and the dynamics of the renal renin-angiotensin system. 1. Tipnis S.R. et al. (2000) J. Biol. Chem 275:33238

## Tight Junctions (2584-2604)

2584

### A Role for JAM-A in the Development of Hepatic Polarity

L. T. Braiterman,[1] S. Heffernan,[1] A. Sharma,[1] M. Herman,[1] A. Khalsa,[1] U. Naik,[2] A. L. Hubbard[1]; [1]Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, [2]Biological Sciences, University of Delaware, Wilmington, DE

The WIF-B cell is a well-characterized, polarized rat-human hybrid line with properties similar to hepatocytes *in vivo*. The presence of apical "cysts" that are sealed by functional tight junctions (TJ) between adjacent cells is a hallmark of the mature WIF-B hepatic phenotype. Development of this phenotype is biphasic over 14 days in culture: cells first exhibit a simple columnar epithelial morphology with TJ proteins forming a belt around the cell apex (~6-9 d); later, cells exhibit the hepatic phenotype where TJ proteins form a belt around the apical cyst (~9-14 d). Our current goal is to determine the functional relevance of junctional adhesion molecule (JAM) in the phenotypic switch. By RT-PCR, we determined that JAM-A, B and C are expressed in rat liver hepatocytes while only JAM-A is expressed in WIF-B cells. Therefore, day 9 WIF-B cells were infected with recombinant adenovirus encoding human wild-type (wt) JAM or mutant JAM-A and analyzed 4 days later. Cells over-expressing wtJAM-A exhibited the hepatic phenotype to a much lesser extent than those over-expressing a mutant JAM-A that lacked its PDZ binding motif (4 C-terminal residues). Interestingly, both exogenous JAM-A proteins were found at cell-cell contact sites. While neither E-cadherin nor occludin colocalized with either over-expressed JAM-A, ZO-1 co-localized with wtJAM-A to a greater extent than with mutant JAM-A at these sites. Our data suggest that JAM-A may play a role in the development of hepatic polarity. We are currently assessing the effects of the two JAM-A proteins on 6-10 day WIF-B cells and Caco-2 cells, where the simple epithelial cell morphology predominates.

2585

### Regulation of Epithelial Tight Junction Assembly by AMP-activated Protein Kinase

B. Zheng, L. C. Cantley; Division of Signal Transduction, Beth Israel Deaconess Medical Center and Department of Systems Biology, Harvard Medical School,, Boston, MA

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/therine kinase that plays important roles in maintaining cellular energy balance. The activity of AMPK is modulated both by the cellular AMP to ATP ratio and by upstream kinases. Upon activation, AMPK increases energy supply by stimulating ATP-producing pathways and inhibiting ATP-consuming pathways. Recently, one of the AMPK upstream kinases was identified as LKB1, a homolog of C. elegans and Drosphila Par-4 proteins that are essential to the establishment of cell polarity during embryogenesis. Here we have used MDCK epithelial cells as a model system to investigate whether AMPK, as a substrate of LKB1, plays a role in the regulation of epithelial polarization. We found that AICAR, an activator of AMPK, promoted the assembly of tight junctions as indicated by measurement of trans-epithelial resistance (TER) and immunofluorescence analysis of ZO-1 translocation after calcium switch. Expression of a kinase-dead mutant of AMPK (D157A) inhibited the development of TER and Zo-1 translocation after calcium switch. Furthermore, the levels of AMPK phosphorylation (T172) increased during calcium-induced cell polarization, and this increase was dependent on the kinase activity of LKB1, because expression of a LKB1 kinase dead mutant (K78I) but not the wild-type abolished the increase of phospho-AMPK level. These results together support a role of AMPK in the regulation of epithelial tight junction assembly, and provide an intriguing link between cellular energy status and tight junction assembly in epithelial cells.

2586

### Activation of AMPK is Associated with Tight Junction Formation and Maintenance of Epithelial Polarity

L. Zhang,[1] J. Li,[2] L. H. Young,[2] M. J. Caplan[1,3]; [1]Department of Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Department of Internal Medicine (Section of Cardiovascular Medicine), Yale University School of Medicine, New Haven, CT, [3]Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT

AMP activated protein kinase (AMPK), known as a sensor of cellular energy status, is a substrate for LKB1, a kinase capable of polarizing single intestinal epithelial cells. However, it is not clear whether AMPK is also involved in epithelial cell polarization. Using an antibody specific for phospho-Thr172-AMPK, the phosphorylation of AMPK was found to increase 3-fold when MDCK cells regenerate polarity after being depolarized by $Ca^{2+}$ depletion. In repolarizing MDCK cells, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which makes AMPK a

better substrate for LKB1, facilitates tight junction organization under conditions of normal $Ca^{2+}$ concentration, and initiates tight junction assembly in the absence of $Ca^{2+}$. Specifically, under conditions of normal $Ca^{2+}$ concentration, the recruitment of ZO-1, a tight junction protein, to the tight junction region, is faster in the presence of AICAR. In the absence of $Ca^{2+}$, a slow but detectable ZO-1 recruitment to the tight junction region could be observed in the presence, but not in the absence, of AICAR. The activation of AMPK appears to protect epithelial cells at least in part from losing polarity under conditions of energy depletion. The internalization of a basolateral marker Na, K-ATPase could not be detected in the MDCK cells pretreated with AICAR prior to energy depletion induced by 2-deoxy-D-glucose; however, a considerable amount of Na, K-ATPase was internalized in cells that were not pretreated with AICAR before the energy depletion. Taken together, these results suggest that AMPK may contribute to the regulation of epithelial cell polarity.

2587

**PKCζ Activity is Essential for the Establishment of Cell Polarity during the Assembly of Mammary Epithelial 3D- Acini**

L. Thornton, E. McArdle, E. Gilligan, J. Murtagh, F. Martin; Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Ireland

Dispersed primary mouse mammary epithelial cells organize as spherical acini when grown on a laminin-rich ECM. It has been demonstrated that acinus formation begins with the apicobasal polarization of the outer cells of the assembly and subsequent to this, follows cell cycle withdrawal and loss of the internal cells by apoptosis. We have shown that JNK activity is necessary for the establishment of cell polarity and functional acinus formation. We have now investigated the downstream targets of JNK that contribute to the polarisation of the cells in the acini. It is proposed that polarization in an epithelial sheet is established by the generation of an active ternary signaling complex consisting of the atypical PKCζ and two PDZ domain proteins, Par3 and Par6 with resultant activation of PKCζ. We demonstrate that PKCζ is phosphorylated (at Thr410) and active in the early stages of acinus formation in a JNK-dependent fashion. In addition, inhibition of PKCζ activity was sufficient to disrupt the assembly of properly polarized acini. We also show that phospho-[Thr410]-PKCζ distributes with ZO-1 to tight junction sites and is in a complex with Par3 and Par6 in maturing acini; these events can be disrupted by JNK inhibition. In contrast to phospho-PKCζ, the main cellular PKCζ component distributes to a perinuclear location as acini form but this is also dependent on the correct establishment of cell polarity. Furthermore, we show that Par3 co-localises with phospho-PKCζ and ZO-1 to areas of tight junction formation. Finally, we demonstrate that PKCζ activation lies downstream of JNK activation; we therefore propose that JNK activity underpins key molecular events leading to the activation of PKCζ, which is necessary for cell polarisation, a determining event in mammary epithelial acinus assembly.

2588

**A Beta Subunit Specific, Ion-transport Independent Function of the Na,K-atpase is Required for Barrier Junction Formation and Epithelial Morphogenesis in the *Drosophila* Tracheal System**

S. M. Paul, Z. Liang, G. J. Beitel; BMBCB, Northwestern University, Evanston, IL

Although epithelial tubes are essential for the functions of many vertebrate and invertebrate organs, the cellular and molecular mechanisms that control the sizes and shapes of epithelial tubes are poorly understood. Using the Drosophila tracheal (airway) system as a model, we previously demonstrated that the occluding junction in flies, the septate junction (SJ), plays a critical role in epithelial tube size regulation. Mutations in SJ components cause tracheal tubes to become too long and have diameter abnormalities. Importantly, the occluding function of SJ was separable from its tube-size control function. We also unexpectedly found that the Na,K-ATPase localizes to and is required for normal tube-size control and for SJ formation. We now show that the canonical ion pump function of Na,K-ATPase is dispensable for tube-size control and junction formation, thus revealing a novel ion-transport independent function of the Na,K-ATPase. This ion-transport independent activity appears to reside with the beta subunit of the Na,K-ATPase complex since all alpha subunit isoforms tested have tube-size and septate junction activity, but only one of the three beta subunit loci in the Drosophila genome, nrv2, is necessary and sufficient for tube-size control and SJ formation. Chimera analysis between nrv2 and another Na,K-ATPase beta subunit reveals that the unique tube-size and junctional activity localizes to the extracellular domain of nrv2. We will discuss models for the role of Na,K-ATPase in junction formation and of SJs in epithelial morphogenesis, as well as possible evolutionary conservation of these mechanisms.

2589

**Na,K-ATPase is a Novel Member of the Apical Junctional Complex**

S. A. Rajasekaran,[1] S. P. Barwe,[1] S. Ryazantsev,[2] E. E. Schneeberger,[3] A. K. Rajasekaran[1]; [1]Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, [2]Biological Chemistry, University of California, Los Angeles, Los Angeles, CA, [3]Molecular Pathology Unit, Massachusetts General Hospital East, Charlestown, MA

Polarized epithelial cells form a permeability barrier between two biological compartments and have a unique structural organization with distinct apical and basolateral plasma membranes separated by tight junctions. Tight junctions not only give rise to the polarized distribution of receptors, ion channels, transporters and pumps, but also regulate the paracellular solute and water flux. They are multiprotein complexes composed of transmembrane proteins that are linked to the actin cytoskeleton through plaque proteins that also serve as adapters for the recruitment of cytosolic signaling molecules to integrate diverse processes such as cell polarity, cell proliferation, and tumor suppression. Na,K-ATPase is a transmembrane protein consisting of a non-covalently linked α- and β-subunit and catalyses an ATP-dependent transport of three sodium ions out and two potassium ions into the cell per pump cycle, thereby generating a transmembrane sodium gradient across the plasma membrane. More recent evidence suggests that Na,K-ATPase also plays a role in a variety of signaling processes in epithelial cells. In a series of earlier studies we have shown that Na,K-ATPase function is necessary to form and maintain functional tight junctions. We now provide evidence that Na,K-ATPase is localized to tight junctions and is in a complex containing PP2A, annexin II and occludin. The enzyme activity of Na,K-ATPase modulates this complex formation in polarized epithelial cells. Together these results suggest that Na,K-ATPase, PP2A, annexin II and occludin form a novel apical junctional complex involved in the regulation of tight junction permeability in epithelial cells.

2590

**The Coxsackie and Adenovirus Receptor (CAR) is an In Vivo Marker for Epithelial Tight Junctions, Potentially Regulating Permeability**

**and Tissue Homeostasis**

E. Raschperger,[1] J. Thyberg,[2] S. Pettersson,[3] J. Fuxe,[4] R. F. Pettersson[1]; [1]Ludwig Institute for Cancer Research, Karolinska Institute, Stockholm, Sweden, [2]Department of Cell and Molecular Biology (CMB), Karolinska Institute, Stockholm, Sweden, [3]Microbiology and Tumor Biology Center (MTC), Karolinska Institute, Stockholm, Sweden, [4]Cardiovascular Research Institute, University of California, San Francisco (UCSF), San Francisco, CA

Cell adhesion molecules are components of epithelial and endothelial tight junctions, a complex membrane structure that is important for paracellular barrier function and cell polarity. One such component is CAR, a transmembrane protein and a member of the CTX family of Immunoglobulin Superfamily (IgSF) molecules. The role of CAR as a virus receptor has been extensively explored in various adenovirus-based gene transfer/therapy studies but its physiological role, however, has remained less clear. CAR associates with tight junctions in cultured epithelial cells and mediates cell-cell adhesion, and during development, CAR has been shown to be essential for normal cell differentiation and tissue maturation. Here we show that CAR is exclusively expressed in epithelial cells lining the body cavities, such as the gastrointestinal tract, the respiratory system, the bile ducts, the urinary system etc. In all tissues examined, CAR specifically co-localized with the tight junction markers ZO-1 and occludin. No CAR expression was detected in the vasculature, not even in brain capillaries, where endothelial tight junctions are well developed and form the blood brain barrier. A mutant form of CAR, lacking the intracellular tail, was mislocalized in cells when transgenically expressed in mice. Instead of being targeted to tight junctions this mutant CAR protein was diffusely localized over the plasma membrane, confirming the importance of the cytoplasmic portion of CAR for its correct localization and function. Moreover, the expression pattern of CAR in different epithelial cell layers correlated positively with the degree of tight junction maturity and inversely with permeability. Thus, we conclude that CAR, in adult tissues, restrictively localizes to epithelial tight junctions where it may participate in the regulation of epithelial permeability and tissue homeostasis.

2591

**Occludin Phosphorylation and Regulation of Vascular Permeability**

J. M. Sundstrom,[1] B. A. Stanley,[2] D. A. Antonetti[1]; [1]Cell and Molecular Physiology, Penn State College of Medicine, Hershey, PA, [2]Section of Research Resources, Penn State College of Medicine, Hershey, PA

**Objective:** Our objective is to better understand the mechanisms which modulate vascular permeability in health and disease. Recent research has begun to define the molecular mechanisms in diabetic retinopathy which lead to enhanced vascular permeability. VEGF is elevated in the eye in diabetes and acts through protein kinase C to cause the phosphorylation of a transmembrane tight junction protein, occludin, on multiple sites. Preventing this phosphorylation with PKC inhibitors reduces VEGF induced vascular permeability. Therefore, mapping these phosphorylation sites is a critical step in understanding the pathophysiology of increased vascular permeability in diabetic retinopathy. **Methods**: Bovine retinal endothelial cells (BREC) were treated with VEGF for 15min, occludin was immunoprecipitated and separated by SDS-PAGE. Occludin bands were excised, digested with trypsin, and analyzed by MALDI TOF mass spectrometry to identify sites of occludin phosphorylation. Phosphopeptides were identified based on mass accuracy, repeat observations, identification in multiple overlapping fragments, presence in VEGF treated and gel shifted (hyperphosphorylated) occludin bands. Finally, conservation of serine and threonine residues and kinase consensus motifs were used to identify potential phosphorylated residues. **Results**: Six putative phosphopeptides were identified within occludin. This data led to the successful creation of a phospho-specific antibody to Ser490. Immunoblotting and immunocytochemistry experiments have confirmed that Ser490 is phosphorylated in vivo and that VEGF stimulation of BREC increase Ser490 phosphorylation. **Conclusion**: To our knowledge, this is the first demonstration of site specific occludin phosphorylation within cells and provides further evidence that occludin phosphorylation is associated with alterations in vascular permeabilily. Ser 490 is located within the cytoplasmic coiled-coil domain of occludin known to interact with ZO-1 and phosphorylation of this site may alter this interaction. Future experiments, including mutational analysis, will identify the funcitonal significance of occludin phosphorylation.

2592

**Glucocorticoids Increase Barrier Properties of Retinal Endothelial Cells Through Transactivation of Tight Junction Genes**

E. A. Felinski, D. A. Antonetti; Cellular & Molecular Physiology, Penn State College of Medicine, Hershey, PA

Tight junctions provide the barrier between adjacent epithelial and endothelial cells that allows compartmentalization of tissues. In brain and retina, tight junctions between endothelial cells of blood vessels create the blood-brain and blood-retinal barrier. Breakdown of the retinal tight junction complex, and formation of leaky blood vessels, is problematic in several disease states including diabetic retinopathy. Glucocorticoids have been shown to restore and/or preserve the endothelial barrier to paracellular flux, although the mechanism remains unclear. Previous work has shown an increase in content of the tight junction protein occludin in cultured bovine retinal endothelial cells upon glucocorticoid treatment, co-incident with an increase in the barrier properties of cell monolayers. The objective of this study was to determine the mechanism by which glucocorticoids induce the expression of endothelial tight junction proteins. Glucocorticoids increased the expression of endothelial caludin-5 as well as occludin, decreased the expression of claudin-1 and did not change the content of ZO-1. Glucocorticoids increased the promoter activity of the occludin and claudin-5 genes in primary retinal endothelial cells using luciferase reporter constructs for each promoter. The glucocorticoid-mediated increase in occludin protein content and endothelial barrier properties was reversed with both the chemical glucocorticoid receptor (GR) antagonist RU486 and siRNA specific for GR. The GC responsive element has been mapped to a 450 bp occludin promoter fragment and neither this sequence nor the claudin-5 promoter contains a canonical glucocorticoid response element (GRE). These data suggest a mechanism for glucocorticoid induction of vascular endothelial barrier properties that involves up regulation of the genes for transmembrane tight junction proteins occludin and claudin-5 and that the GR is required for this process possibly functioning through the stimulation of a transacting factor that directs occludin and claudin-5 gene transcription.

2593

**Side-by-side and Head-to-head Interaction of Claudin-5 in *Vitro* and in *Vivo***

I. E. Blasig,[1] L. Winkler,[1] N. Zuleger,[1] M. S. Bal,[1] H. Wolburg,[2] G. Krause,[3] K. Gast,[4] J. Piontek[1]; [1]Signal Transduktion, Forschungsinstitut für molekulare Pharmakologie, Berlin, Germany, [2]University Tuebingen, Tuebingen, Germany, [3]Forschungsinstitut für molekulare Pharmakologie,

Berlin, Germany, [4]University Potsdam, Potsdam, Germany
Claudin-5 is known to tighten the paracellular cleft of brain capillary endothelial cells against solutes ≤800 Da and, hence, the blood-brain barrier (BBB). However, the interaction mechanism is unknown and was studied in this investigation. The recombinant segment of the second extracellular loop (2ECL) was detected as dimer by size-exclusion chromatography. The dimer is concentration dependent and sensitive to arginine. Thus, occludin may self-associate via itsthe second extracellular loop may contribute to claudin-5 self-association. We hypothesize that, in the BBB, the dimerization is involved in the attraction of the cell membranes of two adjacent cells (head-to-head interaction) and/or in the strand formation within the cell membrane of one cell (side-by-side interaction). To study the 'side-by-side' hypothesis tight junction-free HEK cells were cotransfected with the fluorescent fusion proteins claudin-5-CFP and claudin-5-YFP. Fluorescence resonance energy transfer (FRET) between the fluorophores demonstrated 'side-by-side' interaction. The FRET signal was not prevented by the mutations of the 2ECL tested so far which would not support 'side-by-side' association of claudin-5 via its 2ECL. Coimmunostaining of mixed cultures of claudin-5-FLAG and claudin-5-YFP showed enrichment of the staining in cell-cell contact areas which was abolished after mutations within the 2ECL. The latter finding indicates that the 2ECL of claudin-5 might contribute to the 'head-to-head' association of claudin-5 between the cells and, consequently, to the paracellular tightness. In general the results makes a contribution to a better understanding of the molecular structure and function of the major tightening protein of the BBB claudin-5.

2594

**Extracellular Domains of Claudin-7 Affect the Paracellular Chloride Conductance in LLC-PK1 Cells**

M. Alexandre, Y. H. Chen; Anatomy and Cell Biology, East Carolina University Brody School of Medicine, Greenville, NC
Tight junctions form selective paracellular diffusion barriers regulating the passage of ions and solutes across epithelia. Claudins are the major structural and functional components of tight junctions and are considered the best candidates for forming paracellular channels. Our recent study demonstrated that overexpression of claudin-7 in LLC-PK1 cells affected the ionic charge selectivity through a concurrent decrease in the paracellular conductance to $Cl^-$ and an increase in the paracellular conductance to $Na^+$. To investigate the importance of the extracellular domains of claudin-7 in the paracellular charge selectivity, we created claudin-7 mutants by reversing the negative charge to a positive charge of selected amino acids located on the extracellular domains using the site-directed mutagenesis method. Claudin-7 mutants were expressed in cultured LLC-PK1 cells and assessed for transepithelial electrical resistance (TER) and dilution potentials. Expression and localization of claudin-7 mutants were determined by immunoblotting and immunofluorescence microscopy. Ectopically expressing of claudin-7 mutants did not change the expression and localization of endogenous tight junction proteins. However, TER and dilution potential measurements revealed that the paracellular $Cl^-$ conductance was significantly increased on the cells expressing claudin-7 mutants compared to that of control cells. This result indicates that the extracellular domains of claudin-7 are critical in determining the paracellular $Cl^-$ permeability in kidney epithelial cells.

2595

**Permeability Barrier Dysfunction in Transgenic Mice Overexpressing a Cytoplasmic Tail Deletion Mutant of Claudin 6**

K. Turksen, T. Troy; Ottawa Health Research Institute, Ottawa, ON, Canada
A defective epidermal permeability barrier (EPB) in premature birth remains a leading cause of neonatal death as a result of its associated complications, which include poor temperature stability, infection by microorganisms through the skin and the outflow of water. Recently we defined a crucial role for Claudins (Cldns), a new superfamily of tight junctional molecules, in the formation of the EPB by engineering transgenic mice with Claudin 6 (Cldn6) overexpressed in the suprabasal layers of epidermis via the involucrin (Inv) promoter. Homozygous mice overexpressing Cldn6 exhibit a perturbation in the epidermal differentiation program leading to a defective EPB and dehydration-induced death within 48 h of birth [Turksen, K., Troy, T.C., 2002. Permeability barrier dysfunction in transgenic mice overexpressing Claudin 6. Development 129, 1775-1784]. To further address the role of Cldns in epidermal differentiation and formation of the EPB, we have initiated a structure function analysis by generating a transgenic mouse overexpressing a cytoplasmic tail deletion mutant of Cldn6 under control of the Inv promoter. Like their full length Cln6 overexpressing counterparts, the tail deletion mutant mice are also born with an incomplete EPB; however, barrier dysfunction is less severe with some animals surviving up to ~21 days postnatally. Tail deletion transgenics lack hair and histological analysis shows epidermal hyperproliferation. Epidermal differentiation anomalies and barrier dysfunction were manifested biochemically by the aberrant expression of late epidermal differentiation markers, including K1, filaggrin, loricrin, transglutaminase 3, and involucrin. Also the expression profiles of other Cldns in the epidermis are modified. These results provide new insight into the importance of the cytoplasmic tail of Cldn6 in epithelial differentiation as well as EPB formation and function. Supported by the March of Dimes Birth Defects Foundation (USA).

2596

**Gap Junctional Intercellular Communication Participates in the Barrier Function of Tight Junctions of Brain and Lung Capillary Endothelial Cells**

H. Fujita, H. Chiba, K. Nagasawa, T. Kojima, N. Sawada; Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan
Gap-junction plaques are often observed with tight-junction strands of vascular endothelial cells, but the molecular interaction and functional relationships between these two junctions remain obscure. We herein showed that gap-junction proteins connexin40 (Cx40) and Cx43 were colocalized and coprecipitated with tight-junction molecules occludin, claudin-5 and ZO-1 in porcine blood-brain barrier (BBB) endothelial cells. Blockers of gap junctional intercellular communication (GJIC) 18β-glycyrrhetinic acid and oleamide did not influence expression of Cx40, Cx43, occludin, claudin-5, junctional adhesion molecule (JAM)-A, JAM-B, JAM-C and ZO-1, nor their subcellular localization in the porcine BBB endothelial cells. In contrast, these GJIC blockers inhibited the barrier function of tight junctions in cells, determined by measurement of transendothelial electrical resistance and paracellular flux of mannitol and inulin. 18β-glycyrrhetinic acid also reduced the barrier property in rat lung endothelial cells expressing doxycycline-induced claudin-1, but not changed interaction between Cx43 and either claudin-1 or ZO-1, as well as their expression levels nor subcellular distribution. These finding suggest that signaling molecules passed through Cx40- and/or Cx43-based gap-junction channels promote the endothelial barrier function without altering expression and localization of tight-junction components analyzed.

2597

**Activation of p21$^{CIP1/WAF1}$ Gene Expression and Inhibition of Cell Proliferation by Overexpression of Hepatocyte Nuclear Factor-4α**

N. Sakai, H. Chiba, T. Itoh, M. Osanai, T. Kojima, N. Sawada; Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan

The F9 murine embryonal carcinoma cell line provides an attractive system for studying epithelial differentiation and antiproliferative processes. We have recently established F9 cells expressing doxycycline-inducible hepatocyte nuclear factor (HNF)-4α, and shown that HNF-4α triggers the gene expression of tight-junction molecules, occludin, claudin-6 and claudin-7, as well as formation of functional tight junctions and polarized epithelial morphology (Exp. Cell Res. 286, [2003] 288 ). Since these events were very similar to those induced by retinoids, we investigated whether HNF-4α, like retinoid receptors, was involved in the control of cell proliferation. We herein show that HNF-4α up-regulates expression of the p21 gene, but not the p15, p16, p18, p19 or p27 gene, in a p53-independent manner, and inhibits cell growth in F9 cells. Similar results were observed in rat lung endothelial cells, in which expression of HNF-4α is conditionally induced by doxycycline. Furthermore, we demonstrate, by reporter assay, that HNF-4α significantly elevates the transcriptional activity of the p21 promoter. Since, HNF-4α is expressed not only in the liver but also in organs containing epithelial cells, such as kidney, intestine, pancreas and stomach, it might also play critical roles in the regulation of epithelial morphogenesis and proliferation in these organs.

2598

**Behavior of Tight-Junction, Adherens-Junction and Cell-Polarity Proteins during HNF-4α-Iinduced Epithelial Polarization**

H. Chiba,[1] S. Satohisa,[1] M. Osanai,[1] S. Ohno,[2] T. Kojima,[1] N. Sawada[1]; [1]Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan, [2]Department of Molecular Biology, Yokohama City University School of Medicine, Yokohama, Japan

We previously reported that expression of tight-junction molecules occludin, claudin-6 and claudin-7, as well as establishment of epithelial polarity, was triggered in mouse F9 cells expressing hepatocyte nuclear factor (HNF)-4α. Using these cells, we examined in the present study behavior of tight-junction, adherens-junction and cell-polarity proteins, and elucidated the molecular mechanism behind HNF-4α-initiated junction formation and epithelial polarization. We herein show that not only ZO-1 and ZO-2, but also ZO-3, junctional adhesion molecule (JAM)-B, JAM-C and cell-polarity proteins PAR-3, PAR-6 and atypical protein kinase C (aPKC) accumulate at primordial adherens junctions in undifferentiated F9 cells. In contrast, CRB3, Pals1 and PATJ appeared to exhibit distinct subcellular localization in immature cells. Induced expression of HNF-4α led to translocation of these tight-junction and cell-polarity proteins to beltlike tight junctions, where occludin, claudin-6 and claudin-7 were assembled, in differentiated cells. Interestingly, PAR-6, aPKC, CRB3 and Pals1, but not PAR-3 or PATJ, were also concentrated on the apical membranes in differentiated cells. These findings indicate that HNF-4α provokes not only expression of tight-junction adhesion molecules, but also modulation of subcellular distribution of junction and cell-polarity proteins, resulting in junction formation and epithelial polarization.

2599

**Cyclin D1 is Transcriptionally Down-regulated by ZO-2 via an E box**

M. Huerta,[1] B. Muñoz,[1] R. Tapia,[2] L. Gonzalez-Mariscal,[2] E. Lopez-Bayghen[1]; [1]Genetica y Biol. Mol., Cinvestav-IPN, Mexico, Mexico, [2]Fisiología, Biofísica y Neurociencias, Cinvestav-IPN, Mexico, Mexico

ZO-2 is a membrane-associated guanylate kinase (MAGUK) protein present at the tight junction (TJ) of epithelial cells. While confluent monolayers have ZO-2 at their cellular borders, sparse cultures conspicuously show ZO-2 at the nuclei. In accordance with the tumor suppressor role proposed for ZO-2, recent data indicates that cyclin D1 levels are down regulated by ZO-2. Concomitantly a diminishing in cell growth and a cell cycle arrest was observed. We further explore the mechanisms underlying the ability of ZO-2 to modulate cyclin D1 gene transcription. To test if ZO-2 modulates cyclin D1 gene transcription, we co-transfected MDCK cells with full length ZO-2 and a reporter plasmid containing cyclin D1 promoter driving luciferase gene (pXP2-CD1). ZO-2 down regulates cyclin D1 promoter both in sparse and confluent monolayers. We also performed RT-PCR assays to analyze the mRNA cyclin D1 levels in cells that over-expressed ZO-2. The results show ZO-2 diminishes mRNA levels in a dose-dependent manner. Since ZO-2 has several characteristic domains, amino, middle and carboxyl regions were over-expressed, in order to explore their role in cyclin D1 promoter regulation. We observed that the amino fragment is a strong repressor and additionally found that the carboxyl region, which concentrates at the cytoplasm also represses cyclin D1 promoter. Moreover we performed deletion analyses and reporter gene assays employing constructs driven by the cyclin D1 promoter and evaluated its activity under ZO-2 influence. We found that ZO-2 negatively regulates cyclin D1 transcription in a manner dependent on the E box of the cyclin D1 promoter.

2600

**In Epithelial Monolayers the Tight Junction Protein ZO-2 Inhibits Cyclin D1 Expression and Cell Proliferation**

R. Tapia, S. Islas, L. Gonzalez-Mariscal; Physiology, Biophysics and Neuroscience, CINVESTAV, Mexico DF, Mexico

In epithelial MDCK cells, the overexpression of ZO-2 generates a down regulation of cyclin D1 (CD1) protein. Since the latter mediates the progression through G1/S stages of the cell cycle, we analyzed the effect of ZO-2 transfection on monolayers previously synchronized by serum deprivation. In these cells the amount of CD1 protein is higher in sparse than in confluent cultures. A flow cytometric analysis of cells stained with propidium iodide, revealed that the overexpression of ZO-2 induced in synchronized cultures, a blockade at the Go/G1 stage of the cell cycle. Moreover, cells transfected with ZO-2 displayed a significantly lower incorporation of 3H-thymidine, than wild type cells and monolayers transfected with the empty vector. This inhibition of cell proliferation is not accompanied by an increase in apoptosis or necrosis, determined by trypan blue exclusion and an annexin V fluorescence assay. To explore if the degradation of CD1 could be due to an increased degradation of the protein, we evaluated the effect of ZO-2 transfection in cultures treated with the proteosoma inhibitor MG132. In these monolayers we observed no further decrease in CD1 content, thus suggesting that the decreased expression of CD1 generated by ZO-2 overexpression is not related to an increased proteosomal degradation of CD1. Taken together these results suggest that ZO-2 plays a role in cell proliferation by inhibiting CD1 protein expression.

2601

**ZO-2 Downregulation Alters the Gate and Fence Functions of the Tight Junction and Modifies the Monolayer Morphology**

S. Hernandez,[1] B. Chavez,[2] L. Gonzalez-Mariscal[1]; [1]Physiology, Biophysics and Neurociences, CINVESTAV, Mexico DF, Mexico, [2]Experimental Pathology, CINVESTAV, Mexico DF, Mexico

MDCK cells transfected with ZO-2 siRNA, display 45% decrease in ZO-2 mRNA and 65% reduction in ZO-2 protein, with no effect on cell viability or cell proliferation evaluated by trypan blue exclusion and 3H-thymidine incorporation. In these monolayers the expression of the TJ proteins claudin-1 and ZO-1 remained unchanged, while that of occludin diminished by 70%. The culture exhibited a transepithelial electrical resistance similar to that found in control and mock transfected monolayers. In contrast cultures transfected with ZO-2 siRNA showed an increased paracellular flux of 70 kDa dextran, indicating that the gate function of the TJ had been altered. When a fluorescent sphyngolipid was added to the apical surface of the cells, the lipid probe remained at the mucosal side of control and mock transfected cultures. Instead in ZO-2 siRNA treated cells, the fluorescent lipid probe appeared also at the upper portion of the lateral membrane, indicating that the fence function of the TJ had been affected. To further explore the impact of ZO-2 knock down on cell adhesion, the number of cells forming aggregates was observed in a hanging drop assay. ZO-2 siRNA transfected cells displayed a transient decrease in the number of cell aggregates. Semithin sections of ZO-2 siRNA transfected cells showed a profound widening of the intercellular space and frequent growth of cells on top of each other. TEM images reveal the appearance of markedly dilated lateral spaces in ZO-2 siRNA treated cells. Taken together these results indicate that the diminished expression of ZO-2 modifies the gate and fence function of the TJ as well as the monolayer morphology.

2602

**Microtubules Regulate Disassembly of Epithelial Tight and Adherens Junctions**

A. I. Ivanov, I. C. McCall, A. Nusrat, C. A. Parkos; Pathology and Laboratory Medicine, Emory University, Atlanta, GA

Epithelial tight junctions (TJ) and adherens junctions (AJ) regulate cell-cell adhesion, paracellular permeability and cell polarity. TJ and AJ are anchored on cytoskeletal structures such as actin microfilaments and microtubules (MT). While the role of F-actin in junctional biogenesis has been extensively studied, a little is known about the involvement of MT in TJ and AJ formation and disassembly. The role of MT is TJ/AJ disassembly was investigated in epithelial cells that lose cell polarity as a result of an extracellular calcium depletion. Calcium depletion of SK-CO-15 colonic epithelial cells induced disruption and internalization of TJ and AJ and reorganization of perijunctional actin microfilaments into contractile F-rings. MT were observed to accumulated inside of such F-actin rings. Depolymerization of MT with nocodazole significantly attenuated junctional disassembly and F-actin ring formation. MT stabilization with either docetaxel or pacitaxel did not prevent TJ/AJ disassembly but attenuated contraction of F-actin rings and prevented accumulation of internalized junctional proteins into an apical cytosolic compartment. Calcium depletion affected neither the balance between stable (acetylated) and instable (tyrosinated) MT nor phosphorylation status of major microtubule-stabilizing protein tau. Pharmacological inhibition of MT motors kinesins with adenylylimido-diphosphate or aurintricarboxilic acid attenuated formation of F-actin rings and junctional disassembly in calcium depleted cells. The kinesin-1 was specifically enriched at TJ/AJ in polarized SK-CO-15 cells and also colocalized with internalized TJ/AJ proteins in calcium-depleted cells. We conclude that MT play a role in disassembly of TJ/AJ during calcium depletion by regulating formation of contractile F-actin rings and endocytosis of junctional proteins.

2603

**Human T Lymphocyte Trafficking across the Lung Epithelium**

J. C. Porter, A. Hall; Lab of Molecular Cell Biology, University College London, London, United Kingdom

Lung disease is the single greatest cause of morbidity and mortality worldwide, often occurring as a result of uncontrolled inflammation. One pathway for clearing leukocytes from the lung, and so limiting inflammation, is across the bronchial epithelial barrier into the large airways; so called egression or transepithelial migration (TEM). We have developed an assay of leukocyte TEM from basal-to-apical surfaces of the bronchial epithelium, and have used this system to define the adhesion molecules and signaling pathways involved. Bronchial epithelial (BE) cells are cultured as standard or inverted monolayers on Transwell™ filters, allowing access to apical and basal surfaces. The presence of tight junctions is confirmed by microscopy, measurements of trans-epithelial resistance and permeability to dextran. T-lymphocytes, labelled with a fluorescent dye, are placed on one side of the epithelial monolayer. After 90 minutes lymphocytes recovered from the other side of the monolayer can be counted. We show that stimulated BE cells produce a polarised gradient of the T cell chemoattractant CXCL11, from the apical surface. Although very few (<5%) of T cells move across a monolayer of epithelial cells in the absence of a chemotactic gradient, 10-60 % of the T cells migrate in response to CXCL11. Analysis of the adhesion molecules required for TEM shows that LFA-1, CD47 and CD29 on the T cells interact with ICAM-1, CD47 and VCAM-1 on the epithelial cell respectively. We also show that T cell adhesion, even in the absence of migration leads to an increase in epithelial permeability. This permeability change is not sufficient for TEM, and additional epithelial cytoskeletal signaling events are required. We are focusing on the role of the epithelial cytoskeleton, and the Rho-GTPases in co-ordinating these events.

2604

***In vivo* Evidence that Tight Junctions are Functionally Disrupted by Attaching and Effacing Bacteria**

J. A. Guttman,[1] Y. Li,[1] M. E. Wickham,[1] W. Deng,[1] A. W. Vogl,[2] B. B. Finlay[1]; [1]Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada, [2]Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada

The human attaching and effacing (A/E) pathogens, enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC), have long been implicated in the disruption of tight junctions *in vitro*. Within the chromosome of various A/E bacteria is a conserved pathogenicity island called the locus of enterocyte effacement (LEE), which encodes a syringe-like type-III secretion system and effector proteins that are essential for tight junction alteration. Recently, a naturally occurring murine model of these diseases has been developed that uses *Citrobacter rodentium* for infection. We have used this model to investigate whether colonic tight junctions are disrupted *in vivo* during A/E infection. Following 7day oral infections with 4-5X$10^8$ bacteria, the distribution of claudins-1, -3 and -5 in the distal colon was altered when evaluated by immunolocalization. Staining normally found along the lateral sides of the colonocytes was now found in the cell cytoplasm while levels of the proteins in the colon remained unchanged. Moreover, molecular tracers penetrated between cells and into the epithelium in infected tissue, but did not do so in controls where the tracer was confined to the lumen of the colon. This functional disruption of tight junctions is dependent on the LEE encoded effector

protein EspF, but not Map or EspG as suggested by *in vitro* EPEC infections. It also is dependent on the presence of bacteria attached to the colonocytes because mice, which eventually clear the infection, retain normal claudin-3 localization. We conclude that functional disruption of colonic tight junctions occurs in mice during infection with *Citrobacter rodentium* and that this is due partly to an altered distribution of claudins.

## Structure & Function of Membrane Proteins II (2605-2617)

2605

### Membrane Protein Alteration in Thalassemic Red Blood Cell (RBC)

M. S. Soriano,[1,2] H. G. Baylon,[3] A. Caviles,[4] M. D. Enriquez,[1,2] F. F. Natividad[1]; [1]Research and Biotechnology Division, St. Luke's Medical Center, Manila, Philippines, [2]College of Science, De La Salle University, Manila, Philippines, [3]Blood and Marrow Transplant Unit, St. Luke's Medical Center, Manila, Philippines, [4]Dr. Fe Del Mundo Medical Center Foundation, Manila, Philippines

Thalassemia, a disease of imbalance between alpha (α) and beta (β) globin chain synthesis, can result to cell membrane damage and premature RBC destruction. The erythrocyte (RBC) membrane is composed of four peripheral proteins: spectrin, the major protein that interacts with actin, protein 4.1 and ankyrin which play an essential role in determining the shape and deformability of RBC. Disruption of interaction among these components may cause loss of structural and functional integrity of the RBC. This study aims to differentiate the membrane protein content between thalassemic and hematologically normal RBC. Specifically, it looked at the assembly of spectrin in the RBC plasma membrane. Eight thalassemic and five normal peripheral blood samples were processed to isolate plasma, cell lysate and membrane proteins or ghost proteins. Protein concentrations were determined using the Bradford Assay. Spectrin distribution was compared using immunofluorescence assay. The mean protein content in plasma, cell lysate and membrane protein of normal RBC are: 0.56430 ug/ml (± 0.070774), 0.65360 ug/ml (± 0.041165) and 0.24252 ug/ml (± 0.065998) respectively, while in thalassemic RBC mean protein content are: 0.58051 ug/ml (± 0.047102), 0.68788 ug/ml (± 0.077435) and 0.15764 ug/ml (± 0.195612) respectively. Results showed no pronounced difference in total protein content of plasma and cell lysate in hematologically normal and thalassemic RBC (t=0.500, p=0.627, t=0.903, p=0.386 respectively). On the other hand, the total membrane protein of thalassemic RBC is lower than that of normal RBC but not statistically significant (t=-0.925, p=0.375). The spectrin assembly in thalassemic RBC exhibited clumping or uneven distribution while in normal RBC there was a uniform distribution. It is believed that this uneven spectrin distribution in the plasma membrane of thalassemic RBC affects the stability, flexibility and shape of RBC.

2606

### Novel Insights into Binding Properties of Cytoskeletal Proteins 41R and 41G with CD44 in HeLa Cells

P. Gascard,[1] W. Nunomura,[2] M. Parra,[1] Y. Takakuwa[2]; [1]LBNL UC Berkeley, Berkeley, CA, [2]Biochemistry, Tokyo Women's Medical University, Tokyo, Japan

[Introduction] We have reported that the 30kDa domain of $4.1R^{80}$ interacts with CD44, and that these interactions are regulated by $Ca^{2+}$ and calmodulin (CaM). The 135kDa 4.1R isoform ($4.1R^{135}$) and 4.1G have an additional polypeptide, referred to as headpiece (HP), upstream of the 30kDa domain. Preliminary data suggest that the HP region of 4.1R regulates $4.1R^{135}$ interaction with membrane proteins but that it is not the case for 4.1G. In order to verify this hypothesis, we investigated the impact of HP on the binding affinity of two chimera proteins, 4.1RHP+4.1G30kDa (RHPG30) and 4.1GHP+4.1R30kDa (GHPR30), to CD44 and on the distribution of these chimera in HeLa cells. [Materials and Methods] HeLa cells were cultured in RPMI medium containing 10% FCS. Specificity of antibodies to 4.1R, 4.1G, 4.1N and 4.1B has been previously characterized (*Kidney Int.* 63:1321). Antibody to CD44 was obtained from Ancell Co. Ltd. RHPG30 and GHPR30 were expressed as GFP fusion proteins in HeLa cells. Protein-protein interactions were measured using the IAsys system. [Results] (1) Immunoblotting and immunocytochemistry analyses revealed that HeLa cells expressed $4.1R^{135}$, 4.1G and 4.1N but not 4.1B; (2) both $4.1R^{135}$ and 4.1G distributed at the cell-cell contact of HeLa cells; (3) both RHPG30 and GHPR30 chimera proteins bound to CD44cyt with similar affinity ($K_{(D)} \sim 10^{-7}$M); (3) both chimera interacted with CaM with similar affinity ($K_{(D)} \sim 10^{-7}$M) in a $Ca^{2+}$-independent manner; (4) $Ca^{2+}$/CaM decreased the binding affinity of both chimeras to CD44 from $\sim 10^{-7}$ M to $\sim 10^{-6}$M; (5) GFP-GHPR30 distributed at a much higher extent to cell-cell contacts than GFP-RHPG30 in transfected HeLa cells. [Conclusion] The results support that HP in $4.1R^{135}$ and 4.1G may specifically regulate binding of their respective 30kDa domain to plasma membrane.

2607

### Role of the Headpiece Region on Cytoskeletal Proteins 41R and 41G Interactions with Red Cell Membrane Proteins

W. Nunomura,[1] P. Gascard,[2] M. Parra,[2] M. Narla,[3] Y. Takakuwa[1]; [1]Biochemistry, Tokyo Women's Medical University, Tokyo, Japan, [2]LBNL UC Berkeley, Berkeley, CA, [3]NYBC, New York, NY

[Introduction] We have reported that the 30kDa domain of $4.1R^{80}$ interacts with Band3 and Glycophorin C (GPC), and that these interactions are regulated by $Ca^{2+}$ and calmodulin (CaM). The 135kDa 4.1R isoform 4.1R ($4.1R^{135}$) and 4.1G have an additional polypeptide, referred to as headpiece (HP), immediately upstream of the 30kDa domain (R30 and G30). Preliminary data suggest that the HP region of 4.1R regulates $4.1R^{135}$interaction with membrane proteins but that it is not the case for 4.1G. To clarify the function of HP, we compared the binding profiles of two chimera proteins, 4.1RHP+4.1G30kDa (RHPG30) and 4.1GHP+4.1R30kDa (GHPR30), to various red cell membrane proteins. [Materials and Methods] Recombinant proteins were expressed as GST fusion proteins except full length $4.1R^{135}$ and 4.1G which were expressed as non-tagged recombinant proteins. CaM was purified as previously described (*JBC*(2000) **275,** 6360). Protein-protein interactions were measured using the IAsys system. [Results] (1) All chimera proteins bound to cytoplasmic domains of Band3 and GPC with similar affinity ($K_{(D)} \sim 10^{-7}$ M) to R30 and G30; (2) RHP30 and GHP30 bound to CaM with a higher affinity ($K_{(D)} \sim 10^{-8}$ M) than chimera proteins ($K_{(D)} \sim 10^{-7}$ M), (3) RHP30 and GHP30 binding to CaM was $Ca^{2+}$-dependent, while binding of RHPG30 and GHPR30 was independent; (3) $Ca^{2+}$/CaM reduced the binding affinity of RHPG30 to Band3cyt and GPCcyt but not that of GHPR30. [Conclusion] Our results support that HP in $4.1R^{135}$ and 4.1G may specifically regulate their respective 30kDa domain binding to membranes. [Acknowledgements] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education of Japan 15570123 to WN and by NIH grant DK56355 to PG. [Note] WN and PD contributed equally to this work.

2608

**Functional Analysis of Zebrafish (*Danio rerio*) Protein 41R Binding to Membrane Proteins and Calmodulin**

W. Nunomura,[1] K. Murata,[2] Y. Takakuwa[1]; [1]Biochemistry, Tokyo Women's Medical University, Tokyo, Japan, [2]Animal Science, UC Davis, Davis, CA

[Introduction] We have reported that the binding profiles of 30kDa domain of human protein 4.1R (H•R30) with membrane proteins, Band3, Glycophorin C (GPC) and p55. A complex of $Ca^{2+}$/ and calmodulin (CaM) regulated 4.1R interactions with those membrane proteins (*JBC* (2000) **275:** 24540). The amino acid sequence of zebrafish (ZF, *Danio rerio*) 30kDa domain of 4.1R (ZF•R30) was 59% conserved that of human. In this study, we studied on the ZF•30 binding to membrane proteins and CaM. [Materials and Methods] Expression of recombinant proteins, H•R30, ZF•R30 (cDNA was gift from Prof. Zon, LI, Dana-Farber Cancer Institute, Boston), cytoplasmic tails of Band 3 (Band 3cyt), GPC (GPCcyt) and p55 of human, and purification of CaM were performed according to previous reports (*JBC* (2000) **275:** 24540). $PIP_2$ was obtained from Sigma. Inside-out-vesicles (IOV) was prepared from human erythrocytes. Protein-protein interaction was measured using IAsys based on the resonant mirror detection method. [Results] (1) ZF•R30 co-precipitated with IOV; (2) The $K_{(D)}$ values of ZF•R30 binding to Band 3cyt and GPCcyt were ~$10^{-6}$ M and ~$10^{-7}$ M, respectively; (3) The $K_{(D)}$ value for ZF•R30 binding to CaM was ~$10^{-7}$ M in the presence and the absence of $Ca^{2+}$; (4) $Ca^{2+}$/CaM did not reduce the binding affinity of ZF•R30 to Band3cyt and GPCcyt; (5) $PIP_2$ inhibited CaM binding to H•R30 but not ZF•R30 in the absence of $Ca^{2+}$. [Conclusion] Our results suggest that $Ca^{2+}$/CaM does not affect on ZF•R30 binding to membrane proteins regardless of $Ca^{2+}$ level. [Foot Note] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education of Japan 12680702 and 15570123 to WN. The authors WN and KM contributed equally to this work.

2609

**Functional Characterization of Cytoskeletal Protein 41R Interaction with Na+/H+ Exchanger NHE1**

W. Nunomura,[1] S. P. Denker,[2] B. A. Watts,[3] V. Calinisan,[4] S. Grinstein,[5] J. Orlowski,[6] C. Brugnara,[7] N. Mohandas,[8] D. W. Good,[3] P. Gascard[4]; [1]Biochemistry, Tokyo Women's Medical University, Tokyo, Japan, [2]UCSF, San Francisco, CA, [3]Medicine and Neuroscience and Cell Biology, UTMB, Galveston, TX, [4]Genome Biology, LBNL, Berkeley, CA, [5]Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, [6]Physiology, McGill University, Montreal, PQ, Canada, [7]Laboratory Medicine, Children's Hospital Boston, Boston, MA, [8]Red Cell Physiology, New York Blood Center, New York, NY

We have previously shown that three members of the family of cytoskeletal 4.1 proteins exhibit segment-specific expression in mouse kidney (Ramez et al., Kidney Int. 2003, 63:1321). In particular, 4.1R expression is restricted to the thick ascending limb (TAL). Strikingly, 4.1R null mouse red blood cells (RBCs) display a dramatic increase in NHE1 activity that may result from a loss of regulation of NHE1 by an unidentified phosphatase (De Francheschi et al., Blood 2004, 104:167a). Here we characterize 4.1R/NHE1 interaction in vitro, assess the impact of the absence of 4.1R on ion transport in medullary TAL tubules (MTALs) and investigate potential mechanisms accounting for NHE1 hyperactivity in 4.1R null RBCs. Pull down and IAsys-based resonant mirror detection binding assays show that 4.1R interacts with NHE1 cytoplasmic domain (KD~100-200nM). This interaction involves primarily a Glu-Glu-Asp motif in 4.1R Four.1/Ezrin/Radixin/Moesin (FERM) domain and two positively charged clusters in the juxta-membrane region of NHE1 cytoplasmic domain. Inhibition of basolateral NHE1 by amiloride or nerve growth factor induces cytoskeleton remodeling that secondarily inhibits transepithelial bicarbonate absorption in MTALs (Watts et al., J. Biol. Chem. 2005, 280:11439). This regulation is similar in wild type and 4.1R null mouse MTALs, arguing against a role for 4.1R in controlling NHE1-induced transport regulation in the TAL. Last, we show that 4.1R null and wild type mouse RBCs express similar levels of the phosphatase “Calcineurin Homologous Protein” (CHP), thus ruling out that NHE1 hyperactivity in 4.1R null RBCs results from a lack of CHP. Studies are under way to unveil possible tissue-specific differences in 4.1R/NHE1 protein complex organization. This work was supported by NIH grant DK56355 to PG and by Grants-in-Aid for Scientific Research from the Ministry of Education of Japan 12680702 and 15570123 to WN.

2610

**The Immunochemical Analysis of the Zebrafish (*Danio rerio*) Protein 41R Isoforms**

K. Murata,[1] W. Nunomura[2]; [1]Animal Science, University of California, Davis, Davis, CA, [2]Biochemistry, Tokyo Women’s Medical University, Tokyo, Japan

[Introduction] Human protein 4.1R (H•4.1R) has been identified as the major component of the erythrocyte membrane cytoskeleton. The $NH_2$ terminal 30kDa (FERM) domain of 4.1R binds to the membrane skeletal proteins and calmodulin. H•4.1R has been also found in nonerythroid cells as multiple alternatively spliced forms, however the functions of these molecules have not been clearly identified yet. The primary structure of 4.1R in zebrafish (ZF) (ZF•4.1R) includes the putative functional domains corresponding to FERM and C-terminal (CTD) domains of H•4.1R (*Development* (2002) **129**, 4359). In this study, to understand the function of 4.1R, we used ZF as the model animal and investigated on the expression and localization pattern of ZF•4.1R as the beginning. [Materials and methods] The blood, liver, ovary, and heart samples were obtained from the anesthetized ZF. The synthetic peptides conjugated with bovine serum albumin (BSA), CEHLNLLERDYFGLV (pep5) and CMGLMDENGKTILLRTQEEIFA (pepCTD) of ZF•4.1R (*Development* (2002) **129**, 4359) were immunized with rabbits. The western blot analysis was performed with these antibodies. [Results] In the erythrocytes, both antibodies detected 150, 80, 75, 25kDa proteins. A 40kDa protein in the liver and heart, and a 25kDa protein in the ovary were detected with both antibodies. Interestingly, a 65kDa protein was detected in the ovary, liver and heart with the anti-pepCTD but not with anti-pep5. Anti-pep5 detected 130kDa protein in the liver and 30kDa protein in the ovary but anti-pepCTD did not. [Conclusion] Our data suggest that zebrafish may have multi-isoforms of 4.1R. [Acknowledgements] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education of Japan 15570123 to WN.

2611

**Structural Analysis of Sarcoglycans Reveals Functional Domains Essential for Their Interaction and Plasma Membrane Targeting**

J. Chen, W. Shi, Y. Zhang, Y. M. Chan; Sigfried and Janet Weis Center for Research, Geisinger Clinic, Danville, PA

Mutations in sarcoglycans (SG) have been reported to cause autosomal-recessive limb-girdle muscular dystrophies (LGMD). In skeletal and cardiac muscle, four sarcoglycans (α, β, γ, δ) are assembled into a complex on the sarcolemma via a stepwise pathway that is dependent on the formation of the β/δ-SG core. In this study, we present a detailed structural analysis of sarcoglycans. Our results from proteolysis and co-

immunoprecipitation indicate that their extracellular regions consist of two distinctive functional domains. Deletion studies demonstrate that the N-terminal half of the extracellular domain is primarily involved in sarcoglycan interaction. On the other hand, the C-terminal half regions of β-SG, γ-SG and δ-SG are critical for plasma membrane localization. This domain consists of a highly conserved sequence and an EGF-like repeat motif that are thought to stabilize the tertiary structure of sarcoglycans. Mutations within these structural feat ures often affect sarcoglycan complex assembly and/or localization in our heterologous expression system. In addition, region near the transmembrane domain is essential for both sarcoglycan interaction and targeting to the cell surface. Finally, we provide evidence that the β/δ-SG core associates with the C-terminus of dystrophin. Our results therefore generate important information on the structure of the sarcoglycan complex and the molecular mechanism underlying sarcoglycan mutations in LGMD.

2612

**Characterization of the Binding Site of Small Ankyrin 1, a Protein of the Sarcoplasmic Reticulum, for Obscurin, a Protein that Surrounds Sarcomeres in Striated Muscle**

M. A. Ackermann,[1] A. Kontrogianni-Konstantopoulos,[2] R. J. Bloch[2]; [1]Biochemistry and Molecular Biology, University of Maryland, Baltimore, School of Medicine, Baltimore, MD, [2]Physiology, University of Maryland, Baltimore, School of Medicine, Baltimore, MD

The proteins that organize the intracellular membranes of cells are not well understood. Here, we consider how the membranes of the sarcoplasmic reticulum, (SR), become organized around the contractile apparatus in striated muscle. We hypothesize that two novel binding partners, small ankyrin 1 (sAnk1, ~17 kDa), concentrated in the network SR, and obscurin (~800 kDa), concentrated at the periphery of the M-lines and Z-disks of each sarcomere, mediate this organization. Obscurin has been shown to bind sAnk1 at its nonmodular, C-terminal region. sAnk1 binds to obscurin through a portion of its cytoplasmic sequence (amino acids 61-130), that is partly homologous to the sequence conserved in larger isoforms of ankyrin. We are characterizing the binding site on sAnk1 for obscurin by site-directed mutagenesis and binding assays. We mutated a set of charged amino acids to alanines in a potential amphipathic α-helical region of the conserved portion of sAnk1, and then measured the binding of the mutated proteins in blot overlays and by surface plasmon resonance. Single mutations (K100A, K101A, R104A, K105A, and R108A) reduced binding of the cytoplasmic portion of sAnk1 to the C-terminal region of obscurin in blot overlays. The K100A/ K101A double mutation reduced binding further (80% reduction as seen with surface plasmon resonance), and mutating 4 of these residues eliminated binding completely. Our results suggest that these five charged amino acids contribute to the interaction between sAnk1 and obscurin. Further characterization of their binding sites will provide an understanding of the interaction between sAnk1in the SR and obscurin at the periphery of the contractile apparatus.

2613

**Obscurin Modulates the Organization of Sarcomeres and the Sarcoplasmic Reticulum**

A. Kontrogianni-Konstantopoulos,[1] D. H. Catino,[1] J. C. Strong,[1] S. Sutter,[2] D. W. Pumplin,[3] M. W. Russell,[2] R. J. Bloch[1]; [1]Physiology, University of Maryland, Baltimore School of Medicine, Baltimore, MD, [2]Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI, [3]Anatomy and Neurobiology, University of Maryland, Baltimore School of Medicine, Baltimore, MD

Obscurin (~800 kDa) is a multidomain protein composed of tandem adhesion modules and signaling domains. Obscurin is not present within sarcomeres but intimately surrounds them, primarily around the M-line and Z-disk, where it is appropriately positioned to participate in their assembly and integration with other sarcoplasmic elements. Consistent with this, obscurin interacts with diverse proteins located in distinct subcellular compartments, including small ankyrin 1 (sAnk1), an integral component of the network sarcoplasmic reticulum (SR), titin and sarcomeric myosin. We used small inhibitory RNA (siRNA) technology to reduce the levels of obscurin in primary cultures of skeletal myotubes, to study its role in myofibrillogenesis and the organization of the SR. siRNA-treated myotubes showed a specific and dramatic reduction in the ~800 kDa form of obscurin by reverse transcription-polymerase chain reaction, immunoblotting and immunofluorescence. M-lines and A-bands, but not Z-disks or I-bands, were severely disrupted when the synthesis of obscurin was reduced. sAnk1 also failed to align around the middle of sarcomeres in myotubes infected with siRNA targeted to obscurin. Myosin and myomesin levels were significantly reduced in treated myotubes, but α-actinin was not, suggesting that down-regulation of obscurin destabilizes proteins of the M-line and A-band but not of the Z-disk. Our findings suggest that obscurin is required for the assembly of the M-line and A-band and for the regular alignment of the network SR around the contractile apparatus. Supported by grants from the NIH (RO1 HL075093 to MWR; RO1 HL064304 to RJB) and the Muscular Dystrophy Association (to AK-K, MWR and RJB).

2614

**Lactoferrin Inhibits Trophoblast Proliferaton via Its Receptor**

V. Lopez; Nutrition, UC Davis, Davis, CA

Inflammation and infection are known to result in pregnancy complications. Lactoferrin (Lf) is an iron (Fe) binding protein that increases during infection and binds to its GPI-anchored receptor, LfR, however its biological function has yet to be determined. Fe bound to Lf results in a protein conformational change, which may play an important role in the dynamics of how Lf interacts with its receptor activating downstream signals. We hypothesized that Lf decreases trophoblasts proliferation via MAPK signaling through LfR, which is dependant upon Lf conformation. BeWo cells (human trophoblasts) were treated with Fe-saturated- (holo) Lf or Fe-unsaturated (apo) Lf. LfR localization was visualized with confocal microscopy. Activated MAPK was measured by Western analysis and cell proliferation by the MTT assay. We demonstrated that apoLf binds with higher affinity to LfR compared to holoLf. The binding of apoLf resulted in immediate LfR clustering. Consequently, MAPK was immediately inactivated and cell proliferation was inhibited. In contrast, holoLf resulted in delayed LfR clustering and MAPK inactivation, and cell proliferation was also reduced. These data confirm the differential cell response to apo- and holoLf. These effects also indicate an inhibitory effect on trophoblast proliferation. In conclusion, Lf may have different roles in the placenta depending on its Fe-saturation (conformation) and this potentially could play a role in infertility associated with inflammation and infection. Supported by NIH HD43240.

2615

**Non-muscle Myosin Heavy Chain IIA and Lck Are Involved in CD38 Internalization**

U. Kim, S. Rah, K. Park, M. Im; Biochemistry and Institute of Cardiovascular Research, Chonbuk National University Medical School, Jeonju,

Republic of Korea
CD38 activation in lymphokine-activated killer (LAK) cells involves IL-8-mediated PKG activation and results in an increase of long-lasting intracellular $Ca^{2+}$ concentation ($[Ca^{2+}]_i$) that is critical for migration of LAK cells. However, our previous study has indicated that CD38 is not phosphorylated directly by PKG. In this study, we have examined molecular mechanism of CD38 activation in LAK cells and Jurkat T cells. Non-muscle myosin heavy chain IIA (MHCIIA) was identified as a CD38-associated protein during IL-8 signaling by MALDI-TOF analysis. The IL-8-dependent association of MHCIIA with CD38 was inhibited by an inhibitor of PKG, and the association of both proteins was significantly increased by the treatment with membrane-permeant cGMP, 8-pCPT-cGMPS. Moreover, IL-8-induced formation of cyclic ADP-ribose (cADPR), increase of $[Ca^{2+}]_i$, and migration of LAK cells were substantially inhibited by the treatment with an inhibitor of MHCIIA, blebbistatin. IL-8 treatment of LAK cells resulted in CD38 internalization that was blocked by pre-treatment with blebbistatin. During CD38 internalization, CD38 was colocalized with MHCIIA as well as Lck in LAK cells. Supporting the observations, levels of CD38, MHCIIA, and Lck in lipid rafts were significantly increased by OKT-3 treatment of T cells compared to the control. A synthesized cell permeable peptide $CD38^{11-20}$, cytoplasmic juxtamembrane domain inhibited OKT-3-induced formation of cADPR, increase of $[Ca^{2+}]_i$, and cell proliferation. These findings indicate that CD38 activation in IL-8 or T cell receptor signaling pathway involves the internalization of CD38 and that the internalization is mediated by the interaction with MHCIIA and Lck via cytoplasmic domain of CD38.

2616

### Elucidating the Structural Role of Dnm1 in Mitochondrial Fission

J. A. Mears,[1] E. Ingerman,[2] M. Marino,[1] J. Nunnari,[2] J. E. Hinshaw[1]; [1]LCBB, NIDDK/NIH, Bethesda, MD, [2]Section of Molecular and Cellular Biology, University of California, Davis, Davis, CA

Dynamin family members are large GTPases involved in many cellular processes including budding of transport vesicles, cytokinesis, pathogen resistance and division of organelles. The structure of classical dynamin from humans has been solved using cryo-electron microscopy. Furthermore, the GTPase and pleckstrin-homology domains of the protein are clearly defined in the structure, and a detailed model has been developed for these regions. Our group is currently interested in characterizing the structural features of several dynamin family members to better understand how this family of proteins can accomplish such varied tasks. Therefore, we are studying Dnm1 (homologous to Drp1/Dlp1 in humans), which regulates mitochondrial fission in yeast and self-assembles at sites of mitochondrial membrane constriction. We have shown with electron microscopy that Dnm1 self-assembles *in vitro* into spiral structures in the presence of nucleotide and absence of lipid. It also decorates lipid in the presence or absence of nucleotide, and the resultant Dnm1 tube forms a helical structure similar to classical dynamin. However, the diameter of Dnm1 tubes (between 100 and 200 nm) is much larger than seen with classical dynamin (40 and 50 nm). This larger diameter is consistent with distinct constriction sites observed during mitochondrial fission *in vivo*. Using a variety of microscopic methods, we are working towards determining a high-resolution structure of Dnm1 to better understand how the protein sequence and architecture affects the size and function of dynamins during membrane fission.

2617

### Serotonin Regulates the Plasma Membrane Expression of Serotonin Transporter Protein Via Its Interactions with Rab4

F. Kilic,[1] V. Lupashin,[2] P. van der Sluijs[3]; [1]Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR, [2]Department of Physiology, University of Arkansas for Medical Sciences, Little Rock, AR, [3]Cell Biology, University Medical Center, Utrecht, The Netherlands

Serotonin (5HT) is a platelet-stored vasoconstrictor that also acts as a neurotransmitter. Alterations in the levels of 5HT in the nervous system, or blood plasma are associated with a number of neuropsychiatric disorders, or major heart and kidney diseases, respectively. The actions of 5HT are mediated by different types of receptors, and terminated by a single 5HT transporter (SERT). Although both substrates and inhibitors are capable of regulating transporter distribution in cells, the mechanism by which 5HT can accomplish this task has remained elusive. We first showed that the plasma membrane expression of SERT is regulated by 5HT in a concentration-dependent manner. We investigated the impact of 5HT on other proteins those also interact with SERT. It has been reported that a small GTPase, Rab4 is modified with 5HT covalently at the phosphate binding domain. This modification stabilizes Rab4 at its constitutively active, GTP-bound form which interacts downstream with effector molecules, resulting in actin-myosin cytoskeleton rearrangement and stimulation of membrane trafficking of small vesicles. We have found that Rab4-GTP was co-immunoprecipitated with SERT and that recycling of SERT involves the action of Rab4. Pretreatment with 5HT at high (≥50 μM) concentrations increased SERT-Rab4 co-localization at intracellular compartments. We propose that 5HT controls the density of SERT on the plasma membrane by enabling its interactions with Rab4, and that this interaction regulates membrane trafficking and plasma membrane expression of SERT.

## Membrane Domains (2618-2635)

2618

### Short-Range Dynamics of Lipid Domains in Model Biomembranes

D. J. Mudaliar, R. P. Walvick, A. A. Heikal; Bioengineering, Pennsylvania State University, University Park, PA

Cellular plasma membranes are heterogeneous, dynamic and complex systems that regulate numerous biological processes. Due to the coexistence of a variety of lipid molecules with different melting temperatures and acyl chain lengths as well as proteins, biomembranes exist as different lipid domains characterized by different physical and biochemical properties. Gel phase lipids have rigid, ordered acyl chains while fluid phase lipids are marked by higher levels of unsaturation and higher freedom of motion of acyl chains. An intermediary lipid phase (rafts), which is rich in sphingolipids and cholesterol, has been implicated in important membrane functions. The long-range (microsecond to seconds) biophysical properties of these domains have been investigated in model systems using fluorescent probes that are inherently specific to the lipid phases. Here, we present a new approach for investigating short-range (picoseconds to nanoseconds) lipid-lipid and lipid-tag interactions, at high spatial and temporal resolution, in giant unilamellar vesicles (GUVs) that mimic biomembranes. We have employed two-photon fluorescence polarization and lifetime imaging microscopy for quantitative imaging of the excited-state dynamics and rotational diffusion of fluorescent markers (DiI-C12,

Bodipy-PC, and Alexa-Cholera toxin B) in different lipid domains. We have also developed image processing and data analysis algorithms for modeling these short-range dynamics, which are extremely sensitive to the surrounding environment and molecular conformations, to understand their correlation to lipid domains. For example, the polarization anisotrpy decays of these probes were modeled as wobbling-in-cone motion that quantifies the spatial restriction on the probe exerted by different lipid domains. These results will help filling the current gap of molecular, short-range knowledge of lipid domains in model membrane systems and, ultimately, will be used to correlate lipid phases to the biological functions of biomembranes.

2619

**Patterning Complexity into Biomimetic Lipid Membranes: Toward Understanding Raft Dynamics**

K. Vats, M. Kyoung, E. D. Sheets; Chemistry, Pennsylvania State University, University Park, PA

The relationship between the structure of biomembranes and their biological functions remains elusive, particularly with respect to cholesterol-rich microdomains or “rafts”. We are exploiting a combination of microlithography and quantitative fluorescence microscopy to systematically probe the molecular interactions that lead to lipid domain formation as well as domain dynamics. Complexity is built into supported bilayers by patterning domains via a polymer lift-off approach. We find that the complex bilayers, composed of fluid phase dilauroylphosphatidylcholine (di12:0 PC; $T_m$ = -1°C) and gel phase dipentadecanoylphosphatidylcholine (di15:0 PC; $T_m$ = 33°C), retain their patterns and form a single continuous bilayer, as assessed by quantitative fluorescence microscopy. Using fluorescence correlation spectroscopy (FCS), we observe that fluid phase lipids retain typical diffusion coefficients ($D$ = $10^{-9}$-$10^{-8}$ $cm^2/s$) whether they are deposited as conventional unpatterned, single-component bilayers or as patterned complex bilayers. In addition to FCS, we are combining single particle tracking with imaging to probe the molecular dynamics associated with lipid domains and interfaces at high spatial and temporal resolution. We are also characterizing these patterned membranes with liquid atomic force microscopy to further confirm the results from the fluorescence measurements about the continuity of the biomimetic membranes. Our results with the initial composition of di12:0 PC and di15:0 PC suggest that our approach will allow us to form chemically well-defined domains *within* the context of another chemically well-defined lipid composition, independent of whether lateral phase separation would result in conventional model membranes. Ultimately, we will follow the dynamics of domains and individual lipids within this milieux as a function of cholesterol content to understand how these physical interactions in cholesterol-rich rafts control biological function.

2620

**The Search for Membrane Microdomains (Lipid Rafts) in *Ascidia ceratodes* Sperm Cells**

D. Diaz, R. A. Koch; Biological Science, California State University, Fullerton, CA

Contact between sperm and egg cells initiates reciprocal activation of metabolic and signaling reactions within both gametes. During activation, ascidian sperm cells undergo a morphological change termed mitochondrial translocation. Sperm-egg interactions in ascidians involves events such as adhesion, signaling, and cytoskeletal remodeling that require *N*-acetylglucosaminidases, G-protein coupled receptors, surface-mounted proteases, and integrins. In order for intra-/extracellular activity of all cells to take place, a platform to facilitate protein gathering and assembly must exist. The structure that is becoming more accepted as being this platform is a microdomain at the surface of the cell termed a lipid raft. These lipid rafts are distinguished by having concentrated aggregates of sphingolipids, cholesterol (Ch) and signaling proteins and being non-ionic detergent insoluble. In order to determine if these structures exist in ascidian sperm, the fluorescent labels, filipin and Alexa 555-conjugated cholera toxin (CTX) were used to label cholesterol and sphingolipid domains. These structures were viewed using epi-fluorescence and analyzed using the NIH computer software, ImageJ. Experiments were conducted at 4 °C, 15 °C and 25 °C to ensure that temperature was not influencing labeling behavior (results were not significantly different). Analysis of digital micrographs revealed that high concentrations of Ch and sphingolipids, consistent with the presence of microdomains, increased in number and intensity following sperm activation. Labeled patches translocated with mitochondrial movement. Experiments to study the behavior of microdomains following activation by the G-protein activator, mas-7, and integrin-clustering antibody, mAB-12G10, and possible disruptive effects of methyl-β-cyclodextrin are in progress. Our evidence supports the preliminary conclusion that lipid rafts play a role in signaling processes involved in ascidian sperm activation (Funded by NIH Grant 2R25-GM-56625).

2621

**ABCA1 Expression Disrupts Raft Membrane Microdomains Through its ATPase-Fueled Functions**

Y. Landry,[1] M. Denis,[1] S. Nandi,[1] S. Bell,[1] A. M. Vaughan,[2] X. Zha[1]; [1]Ottawa Health Research Institute and University of Ottawa, Ottawa, ON, Canada, [2]Department of Medicine, University of Washington, Seattle, WA

ATP binding cassette transporter A1 (ABCA1) is an ATP-fueled transporter known to facilitate efflux of both phospholipids and cholesterol to extracellular acceptors, such as apoA-I. How ABCA1 mediates cholesterol export is largely unknown at present. Upon transient induction of ABCA1 expression in BHK cells, we observed the disruption of caveolae-rich plasma membrane lipid rafts. ABCA1-expressing cells also had a higher percentage of non-rafts on the plasma membrane, which was not due to cholesterol redistribution or alterations in cellular free cholesterol content. These observations suggest a re-organization of the plasma membrane by ABCA1. As a member of ancient ABC transporter families, ABCA1 is thought to flip phosphatidylserine (PS) from the inner to outer leaflet of the plasma membrane by hydrolyzing ATP. This raised the possibility that, by flipping PS, ABCA1 may disrupt lipid-lipid interactions, leading to an increase in the non-raft pool of the plasma membrane. This process should then depend on ABCA1’s ATPase activity. To test this hypothesis, we expressed an ABCA1 mutant, A937V, which lacks ATPase activity. This mutant can effectively traffic to the plasma membrane, but is unable to flip PS or export cholesterol. In BHK cells expressing the A937V mutant, we observed that caveolae-rich microdomains remained intact, similar to mock cells. In addition, there was no increase in the non-raft pool by this mutant, in contrast to wild type ABCA1. We thus conclude that ABCA1 re-organizes the plasma membrane by actively countering lipid ordering through an ATPase-fueled function, and speculate that this membrane re-organization is one pre-requirement for cells to efflux cholesterol. Our observations may imply that, in live cells, the microdomains are actively maintained by many events at any given time and that the overall process is likely far from equilibrium, contrary to artificial liposomes.

2622

**Giant Plasma Membrane Vesicles Derived from Mammalian Cells Show Large-Scale Fluid/Fluid Phase Separations and Protein Sorting**

A. T. Hammond,[1] T. Baumgart,[2] P. Sengupta,[1] W. W. Webb,[3] D. Holowka,[1] B. Baird[1]; [1]Chemistry and Chemical Biology, Cornell University, Ithaca, NY, [2]Chemistry, University of Pennsylvania, Philadelphia, PA, [3]Applied and Engineering Physics, Cornell University, Ithaca, NY

We have developed a method for producing giant plasma membrane-derived protein-rich membrane vesicles (GPMVs) that show a pronounced capacity for lateral phase separation. Such large-scale separations may represent exaggerated examples of the lateral membrane heterogeneities that occur in cellular membranes and are hypothesized to act as a mechanism for regulating the interactions of certain classes of membrane proteins. This hypothesis makes the assumption that proteins possess intrinsic properties that allow them to respond to differences in their local membrane environment. We have tested this assumption for a set of proteins involved in the signal cascade of the immune-cell receptor Fc-epsilon-RI from mast cells. Membrane proteins, including both transmembrane and lipid-anchored proteins, do in fact respond to the differences in membrane environment and partition preferentially into one or the other of the coexistent fluid phases. This partitioning is observed by fluorescence microscopy and can be compared to the distribution of fluorescent lipid analogs included in the vesicles, which are collected from RBL mast cells that have been chemically induced to shed these plasma membrane vesicles. The lipid and protein composition of these vesicles is highly complex, and similar but not identical to the RBL cell plasma membrane. A surprising finding is that inner leaflet proteins anchored to the plasma membrane by saturated acyl chains preferentially partition into the less ordered phase of these GPMVs. These vesicles lack an underlying actin cytoskeleton and have lost some of the leaflet asymmetry characteristic of a plasma membrane. These factors may influence the observed phase partitioning and the size of the coexisting domains. Because of the importance of receptor crosslinking in mast cell signaling, the effects of crosslinking signaling molecules in these phase-separated membranes are being examined.

2623

**Newly Biosynthesized Sterols Are Located in a Lipid Raft Domain and Serve as an Important Pool for ABCA1-Mediated Cellular Sterol Efflux**

Y. Yamauchi,[1] P. C. Reid,[1] S. Yokoyama,[2] C. C. Y. Chang,[1] T. Y. Chang[1]; [1]Biochemistry, Dartmouth Medical School, Hanover, NH, [2]Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

We monitored the fate of endogenously synthesized sterols in normal and Niemann-Pick type C cells with respect to their intracellular trafficking, cellular distribution, and efflux. The results show that biosynthesized precursor sterols quickly leave the endoplasmic reticulum (ER) for the plasma membranes, and then move back to the ER, to be converted to cholesterol. The retrograde transport of precursor sterols is largely independent of NPC1 and NPC2 proteins. However, long after biosynthesis (more than 24 hours), small but significant amounts of the precursor sterols finally accumulate in the late endosomes/ lysosomes of the NPC1 and NPC2 mutant cells, but not those of the normal cells. We then analyzed the cellular distribution of newly synthesized sterols in normal cells by using sucrose density gradient centrifugation after treating cells with non-ionic detergents. The results show that most of the newly synthesized sterols are localized in a detergent-insoluble lipid raft domain. This domain is also enriched in the protein ABCA1, which plays an important role in cellular lipid efflux. Our results reveal that newly synthesized sterols are enriched in a lipid raft domain, and serve as an important pool for ABCA1 mediated cellular sterol efflux.

2624

**Domain Formation and $PIP_2$ Segregation in Biomimetic Monolayers**

I. Levental,[1] J. Solon,[2] P. Janmey[2]; [1]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA, [2]Department of Physiology, University of Pennsylvania, Philadelphia, PA

The discovery of sphingolipid and cholesterol-rich detergent-resistant membrane (DRM) fractions in mammalian cells has led to significant inquiry into the structural and functional arrangement of lipids in the plasma membrane. Lipid rafts, as the original DRMs were termed, have been found to be rich in signaling and adhesion molecules, as well as structural and scaffolding proteins. Other lateral heterogeneities containing microdomains of important phospholipids, such as polyphosphoinositides (PPI), have been postulated to be critical for localization and activity of membrane active enzymes. The objective of this study was to develop a system to mimic the non-ideal mixing behavior of biologically-relevant lipids and determine the effect of this behavior on the lateral distribution of $PIP_2$. We have used a Langmuir monolayer as a model system to show liquid-liquid phase separation in mixtures of bio-derived lipids. Specifically, we have observed domain formation in mixtures of 1-palmitoyl, 2-oleyl phosphatidylcholine (POPC), L-α phosphatidic inositol 4,5-phosphate ($PIP_2$), and cholesterol. Domains were visualized using fluorescence microscopy by addition of a fluorescent contrast agent. It was found that mixtures of SOPC, cholesterol, and $PIP_2$ form two immiscible liquid phases. One phase was found to be rich in phospholipids, in particular $PIP_2$. At low cholesterol concentrations (<50mol %), the two phases mixed upon compression at pressures much lower than those speculated to be relevant for biological membranes (~5mN/m). However, at higher cholesterol concentrations, the lateral heterogeneity of the monolayer persisted to much higher pressures, and were not made miscible before collapse of the monolayer (~40mN/m). We conclude that these monolayers are a reasonable model of lipid demixing (i.e. lipid raft and domain formation) in the plasma membrane, and that $PIP_2$ is preferentially excluded from the cholesterol-rich phase, which is analogous to the DRM fractions of biochemical experiments.

2625

**Transient Coupling of Lipid-anchored Raftophilic Molecules in the Inner and Outer Leaflets of the Cell Membrane as Detected by High-speed, Simultaneous Two-color, Single-molecule Tracking**

I. Koyama-Honda,[1,2] K. Ritchie,[3] T. Fujiwara,[1,2] E. Kajikawa,[3] R. Iino,[3] H. Murakoshi,[1,2] A. Yoshimura,[4] T. Kobayashi,[3] A. Kusumi[1,2]; [1]Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, [2]Membrane Mechanisms Project, JST-ICORP, Kyoto, Japan, [3]Department of Biological Science, Nagoya University, Nagoya, Japan, [4]Institute for Bioregulation, Kyushu University, Fukuoka, Japan

Coupling of lipid-anchored molecules between the outer and inner leaflets of the bilayer of the cell membrane is one of the key issues in the studies of raft-mediated signal transduction by GPI-anchored receptors. We addressed this issue by observing the recruitment of H-Ras or Lyn (inner leaflet) to artificially-induced small clusters of a ganglioside GM1 or a GPI-anchored protein CD59 in the outer leaflet in live HeLa cells, using a high-speed, dual-color, single-molecule tracking technique at a time resolution of 6.5 ms. After stimulation in the outer leaflet, i.e., crosslinking by

secondary antibodies of CD59 tagged by Alexa633-anti-CD59 antibody or of GM1 labeled with Alexa633-cholera toxin B, dynamic recruitment of GFP-H-Ras or Lyn-GFP in the inner leaflet to GM1 or CD59 clusters located in the outer leaflet was observed. Clustering of outer-leaflet molecules induced transient colocalization of single molecules of GFP-H-Ras or Lyn-GFP, lasting ~200 ms, in a cholesterol-dependent manner. Meanwhile, crosslinking of a non-raft phospholipid in the outer leaflet did not induce colocalization of inner-leaflet molecules. These results suggest that the engagement of raftophilic molecules in the outer leaflet can recruit beneath their clusters raftophilic molecules in the inner leaflet for short periods (~200 ms), which might explain why immunofluorescence colocalization experiments of fixed cells generally report low and/or variable levels of colocalization. Coupling between the inner and outer leaflets by interdigitating lipids in the raft may be weak, but might be sufficient to induce such transient recruitment of the inner-leaflet molecules. Such a coupling between two leaflets may be responsible for the signal transduction across the bilayer or may assist the signal transduction of raft-associated transmembrane proteins.

2626

### Transbilayer Distribution of a Fluorescent Sterol in Living Cells

M. Mondal, F. R. Maxfield; Biochemistry, Weill Medical College of Cornell University, NY, NY

Cholesterol is an important constituent of mammalian cell membranes and plays a significant role in signaling and membrane trafficking. It is most abundant in plasma membranes and in the endocytic recycling compartment. The transbilayer distribution of sterols is poorly characterized. We have used quantitative fluorescence imaging in living cells to study the transbilayer distribution of dehydroergosterol (DHE), a naturally occurring fluorescent cholesterol analog that mimics many of the properties of cholesterol. Addition of a water soluble quencher, trinitrobenzene sulphonic acid (TNBS), which doesn't cross the bilayer during the time scale of our experiment, shows only 10% quenching of DHE fluorescence on the plasma membrane of the TRVb1 cells. When TNBS was microinjected inside the cells, almost all the DHE fluorescence in the plasma membrane and in the endocytic recycling compartment was quenched within seconds. Similar quenching was found when TNBS was added to cells after permeabilization. Our results indicate that most DHE in the plasma membrane and in the endocytic recycling compartment is in the cytoplasmic leaflet. Since DHE shares many properties with cholesterol, including its distribution among cellular organelles and the ability to form detergent resistant, raft-like membranes, these results raise the possibility that most plasma membrane cholesterol is in the inner leaflet and not in the sphingolipid-rich outer leaflet.

2627

### Two-dimensional Surfactants and Lipid Raft Organization

M. J. Saxton,[1] A. Oldham,[2] P. F. Almeida[2]; [1]Dept of Biochemistry and Molecular Medicine, University of California, Davis, CA, [2]Dept of Chemistry, University of North Carolina, Wilmington, NC

One of the main questions about lipid rafts is why liquid-ordered (Lo) domains in model lipid mixtures are large enough to be observed by light microscopy but domains of the raft phase in cell membranes are submicroscopic. We consider the existence of 2D surfactants in cell membranes and propose experimental tests. These hypothetical molecules would have a "raftophilic" face and a "raftophobic" face. They would tend to bind to the raft-nonraft interface, and to disperse rafts as a microemulsion. The presence of 2D surfactants suggests an organized raft structure in which purely raftophilic species are in the Lo phase, purely raftophobic species are in the liquid-disordered (Ld) phase, and 2D surfactants are at the interface. Candidate species include lipids and GPI-linked proteins with one saturated and one unsaturated acyl chain; acylated proteins with raftophilic and raftophobic chains; and proteins, either peripheral or transmembrane, with raftophobic and raftophilic faces. These species would be permanent 2D surfactants; a less permanent form would be a complex of a raftophilic and a raftophobic species crosslinked by an external ligand. Alternatively, raft size could be determined by a monotopic protein replacing the inner leaflet of the membrane. Preliminary data in majority-Lo mixtures of POPC/sphingomyelin/cholesterol, obtained using fluorescence resonant energy transfer between two probes that partition preferentially into the Ld phase, show a decrease in energy transfer upon addition of the amphipathic peptide mastoparan or annexins A4 and A5, if POPC is the minor component. This decrease is consistent with the possibility that these proteins cause a reduction in Ld domain size. Supported by NIH grants GM-038133 (MJS) and GM-059205 to the University of Virginia (PFA).

2628

### Microbial Glycosylated Phosphatidylinositol Targets Plasma Membrane Lipid Rafts to Interfere with $Ca^{2+}$ Signaling and Membrane Trafficking in Macrophages

I. Vergne, H. Lee, J. Chua, V. Deretic; Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM

*Mycobacterium tuberculosis* is a facultative intracellular pathogen which survives in macrophages by blocking the maturation of phagosomes in which it resides, into fully degradative phagolysosomes. A heavily glycosylated phopshatidylinositol product of *M. tuberculosis*, termed lipoarabinomannan (MtLAM) has been shown to participate in the arrest of phagosome maturation (Fratti et al., (2001), J. Cell. Biol). MtLAM inhibits a $Ca^{2+}$/ hVPS34PI3K cascade, essential for phosphatidylinositol 3-phosphate generation on phagosome thus inhibiting phagolysosome biogenesis (Vergne et al., (2003), J.Exp.Med.). The objective of this study was to determine the mechanism of MtLAM action on signaling and consequently on phagosome maturation. Using detergent-based raft isolation method, we show that MtLAM associates mainly with rafts in macrophages. This association leads to a reorganization of the rafts and to alterations of raft-dependent- signaling events, such as translocation of sphingosine kinase to plasma membrane. Our data suggest that *Mycobacterium tuberculosis*, by shedding lipoarabinomannan, affects processes involved in signaling and trafficking by targeting rafts.

2629

### Nanoscale Signal Transduction Across the Plasma Membrane

Y. Chen, B. Yang, K. Jaconson; Department of Cell and Developmental Biology, UNC at Chapel Hill, Chapel Hill, NC

Previous unpublished studies indicate that cross-linking GPI-anchored cell surface molecules, such as CD59, stabilizes the short-lived "cluster" rafts and increases the size of rafts (K. Suzuki, et al., Biophys. J. Annual Meeting Abstracts, 2002 : 348a) by SFK-mediated attachment of the cluster to the cytoskeleton. Here we use single particle tracking to detect the dynamics of these longer-lived rafts. To achieve optimal cross-linking, we incubated C3H fibroblast cells first with biotinylated anti-Thy1 antibodies, then anti-biotin antibody-coated colloidal gold particles, after which

anti-mouse IgG antibodies were added. Under these conditions, transient anchorage of gold-labeled Thy-1 was observed for periods ranging from 300 ms to 10 seconds. Cholesterol depletion and addition of Src family kinases (SFKs) inhibitors PP2 abolished transient anchorage, suggesting the involvement of both cholesterol and SFKs in cross-linking dependent transient anchorage. Further proof of the involvement of SFKs was obtained from Src, Yes, Fyn defective (SYF) cells, in which transient anchorage was not detected. Caveolin-1 knockout cells showed reduced transient anchorage by 1/3, suggesting the partial participation of caveolin-1. Another GPI-anchored protein, CD73, a 5'-ectonucleotidase, exhibited qualitatively similar behavior in IMR-90 human fibroblasts, indicating that these cross-linking induced effects are general. In addition, in PI3 kinase inhibited IMR-90 cells, short-period transient anchorage (less than 1 second) increased sharp and no long-period anchorage occurred. In addition, short-range directed motion occurred, possibly implicating motor-driven transport. (Supported by NIH GN 41402)

2630

**Lateral Heterogeneity in the Distribution of Lipids and Proteins in Plasma Membranes of Live Cells and Isolated Membrane Vesicles Detected by Fluorescence Resonance Energy Transfer and Confocal Microscopy**

P. Sengupta, A. Hammond, T. Baumgart, D. Holowka, B. Baird; Chemistry & Chemical Biology, Cornell University, Ithaca, NY

We have utilized fluorescence resonance energy transfer (FRET) between carbocyanine lipid analogues in the plasma membrane of RBL mast cells to investigate lateral distributions of lipids and to develop a general method for quantitative measurements of lipid heterogeneity in live cell membranes. FRET measured as fluorescence quenching of long chain donor probes such as DiO-C18 is greater with long chain, saturated acceptor probes such as DiI-C16 than with equimolar concentrations of unsaturated or shorter chain acceptors. Sucrose gradient analysis of plasma membrane-labeled, Triton X-100 lysed cells shows that proximity measured by FRET correlates with the extent of probe partitioning into detergent-resistant membranes. These and other FRET data are consistent with nanometer scale phase separations of lipids in the outer leaflet of the plasma membrane. In addition, sucrose gradient analysis of plasma membrane vesicles indicate that membrane heterogeneity is present even in the absence of the membrane-associated cytoskeleton, as proteins and lipids show differential distribution following detergent lysis. Giant plasma membrane vesicles exhibit large scale liquid order (Lo) and liquid disorder (Ld) phase separation, with napthopyrene (an order preferring probe) and Rhodamine-DOPE (a disorder preferring probe) exhibiting complementary partitioning. Partitioning of putative raft proteins such as GPI-anchored Thy-1 and non-raft proteins such as CD43 were complementary in the phase separated vesicles, with Thy-1 preferring the ordered phase. These results demonstrate that Lo/Ld phase separations coexist in compositionally complex plasma membranes, and the cytoskeleton and associated proteins appear to play an active role in preventing large scale phase separation in live cell membranes.

2631

**Imaging of Viral Diffusion Reveals Insights into the Dynamics of the Plasma Membrane**

C. J. Burckhardt,[1] I. F. Sbalzarini,[2] J. A. Helmuth,[2] H. Ewers,[3] P. Koumoutsakos,[2] U. F. Greber[1]; [1]Institute of Zoology, University of Zürich, Zürich, Switzerland, [2]Institute of Computational Science, ETH Zürich, Zürich, Switzerland, [3]Institute of Biochemistry, ETH Zürich, Zürich, Switzerland

The dynamic organization of the plasma membrane and its receptors has been a long standing puzzle in cell biology and signal transduction. Answering this question is essential to understand how signals from the outside are transmitted to the inside of a cell. Despite years of work to determine how proteins bind each other in a particular signal transduction pathway, the available data do not explain how these proteins meet to interact specifically and successfully. Viral particles are excellent tools to tackle the functional organization of the plasma membrane. Most of the more than 50 human Adenovirus (Ad) serotypes use the Coxsackie virus B Ad receptor (CAR) or the membrane-cofactor CD46 for cell attachment. The entry of the species C Ad serotypes Ad2 and Ad5 involves CAR binding and activation of alpha v integrin coreceptors, clathrin-mediated viral uptake and the induction of macropinocytosis engulfing large amounts of membranes and fluids. In contrast, the functional interactions between CAR and the integrin coreceptors on the cell surface are largely unknown. We will present our recent data documenting surface movements of fluorescently labeled Ad2 using total internal reflection fluorescence microscopy at 50 Hz acquisition frequencies. The data reveal a dynamic structure of the plasma membrane with confined viral motilities owing to CAR, slow directional movements and fast processive movements mediated by activated integrin receptors.

2632

**Clustering of Chemotactic Receptors in E coli with Altered Morphologies**

D. Weibel, I. Schneider, E. Garner, R. D. Vale, S. Khan; The Physiology Course, Marine Biological Laboratory, Woods Hole, MA

E. coli possess a sophisticated signal transduction machinery that involves receptor-mediated binding of extracellular attractants and repellants and an intracellular relay mechanism that control the rotation of the flagellar motor. The signal transduction components are concentrated at one pole of the bacteria. This high degree of spatial order is thought to be important producing a high degree of cooperativity that is necessary for sensing shallow gradients of attractants/repellants. However, the mechanism that produces this pole clustering is unknown. Here, we have visualized a receptor-GFP fusion protein (TAR-GFP) and examined conditions that might affect its clustering. We chemically depolymerized the bacterial actin MreB, which produces spherical instead of rod-shaped cells, and found that receptors remained clustered. We also produced long filamentous bacteria by inhibiting septation, confirming that additional receptor clusters formed along the length of the rods, and these nonpolar clusters often appeared to have regular spacing. Finally, we observed small microclusters of TAR-GFP that diffused rapidly in the membrane. We propose these microclusters might be precursors of larger receptor clusters and that clustering reflects a self-assembly process that does not require the bacterial cytoskeleton and occurs in cells with dramatically different shapes.

2633

**Characterization of Septin7 and Novel Proteins of Compacted Myelin Identified Through Multi-Dimensional Protein Identification (MudPit)**

A. D. Roth, A. Ivanova, D. R. Colman; Montreal Neurological Institute, McGill University, Montreal, PQ, Canada

Axonal ensheathment by myelinating cells produces sections along axons with different electrical resistances: low resistance nodes of Ranvier and myelin-surrounded high resistance internodes. This arrangement allows action potentials to "jump" from one node to the next, resulting in faster

transmission along axons of smaller diameter and lower metabolic costs when compared to non-myelinated axons. Myelin formation, deposition and maintenance is dependent on the establishment of two structurally and biochemically discernible domains: a) compact myelin: multilamellar stacks of plasma membrane sheets, and b) non-compact myelin: cytoplasmic channels that border the compact myelin domains, attach them to the cell body and participate in anchoring the myelin sheath to the axonal membrane. In order to identify proteins involved in the organization of these domains we took advantage of the high lipid content of compact myelin to cleanly separate it from other neural membranes. Proteins were characterized by reverse-phase HPLC coupled to Electro-Spray Double Mass Spectrometry ("MudPit"). 122 proteins were detected and classified: 9 were classic myelin markers including proteolipid protein and myelin basic protein, (which together make up 75% of CNS myelin proteins). The rest of the proteins were: 20% known plasma membrane, 54% cytosolic and 19% mitochondrial. We focused on the septin family of small GTPases because of their involvement in formation of diffusion barriers in mammalian cells and confirmed the presence of Septins 7 and 8 in CNS and PNS myelin. We show that Septin7 is distributed through cytoplasmic channels of myelinated tracts (CNS and PNS) and that its levels increase as sciatic nerves mature. We propose that this protein may be involved in the formation of the sharp border between compacted and non-compacted myelin.

2634

**Partitioning of the Endoplasmic Reticulum during Cell Division**

M. K. Rantanen, H. Vihinen, E. Jokitalo; Electron Microscopy Unit, Institute of Biotechnology, Helsinki, Finland

Partitioning of the major subdomains of the endoplasmic reticulum (ER) - the nuclear envelope (NE), rough and smooth ER - in animal cells during cell division is poorly understood. Electron microscopic data suggests partial fragmentation of the membranes in some cell types. On the other hand, light microscopy shows that after NE breakdown (NEBD) some of NE components disperse into ER which remains continuous throughout the cell division. It is not known, however, whether NE proteins spread evenly throughout ER, or, whether ER network organization itself changes during cell division. NE and peripheral ER of interphase and mitotic Chinese hamster ovary cells were studied with light and electron microscope. Live cells carrying either NE or total ER labels were imaged in three dimensions with the confocal microscope. Three-way-junctions representing the degree of ER networking and tubule lengths of ER profiles in confocal optical sections were quantified. Similarly labelled fixed cells were used to model ER structures by 3D electron tomography. In confocal images, ER of the dividing cells appeared tightly networked and quantification showed there were more three-way-junctions and short tubules than in interphase cells. The labelling pattern of the NE protein lamin B receptor resembled that of total ER labelling after NEBD to the end of anaphase. After that it accumulated on NE surrounding the chromosomes. Electron tomography revealed the continuity of ER in dividing cells. It also confirmed dispersion of the NE label into ER after NEBD. However, the distribution of this label was not homogeneous: stained and unstained ER tubules were directly continuous with each other. These observations suggest the ER organization of the dividing cells is different from that of interphase cells, which may be required to ensure even distribution of ER to both daughter cells.

2635

**mRNA Mis-targeting to ER Subdomains Disrupts Correct Protein Deposition in Rice Endosperm Cells**

H. Washida,[1] A. Sugino,[1] A. J. Crofts,[1] N. Crofts,[1] M. Satoh,[2] M. Ogawa,[3] T. Kumamaru,[2] H. Satoh,[2] T. W. Okita[1]; [1]Institute of Biological Chemistry, Washington State University, Pullman, WA, [2]Faculty of Agriculture, Kyushu University, Fukuoka, Japan, [3]Department of Life Science, Yamaguchi Prefectural University, Yamaguchi, Japan

RNA localization is utilized for the synthesis of distinct classes of storage proteins and their efficient packaging within separate endomembrane compartments in developing rice endosperm cells. Prolamine RNAs are targeted to the spherical protein body ER which bounds the newly assembled prolamine polypeptides whilst glutelin RNAs are enriched on adjacent cisternal ER. Following translocation into the ER lumen, glutelin precursors are routed via the Golgi to the protein storage vacuole (PB-II). This relationship between RNA and protein localization within the endomembrane system exists for all RNAs examined to date with one exception. *in situ* RT-PCR and confocal microscopy analysis revealed that the 26 kD α-globulin mRNA is localized to PB-ER but the protein is not retained within this subdomain. Instead, it is rapidly exported to the Golgi and transported to PB-II. Analysis of RNA targeting mutants in rice provides the cellular basis for this unique relationship between globulin RNAs and their coded proteins. When globulin or glutelin RNAs co-exist on the same ER subdomain due to mis-localization of one of the RNAs, normal protein export from the ER and transport to PB-II are disrupted. Both globulin and glutelin proteins are observed in large dilated ER structures and at the cell's periphery. Hence, localization of globulin and glutelin RNAs to separate ER subdomains is essential for PB-II biogenesis and suggests that globulin and glutelin proteins are transported to PB-II by separate pathways.

## Exocytosis: Regulated Secretion (2636-2651)

2636

**Caveolin-1 is a Novel Regulator of Cdc42 and Targets Granules to Active Fusion Sites in Pancreatic β-Cells**

A. K. Nevins, D. C. Thurmond; Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN

Insulin exocytosis is a tightly regulated process whereby upon stimuli intracellular granules translocate and fuse to the plasma membrane via SNARE proteins. However, the specific targeting of granules to active sites of exocytosis in a stimulus-dependent manner remains unknown. Recent studies have suggested that caveolae function as microdomains for cellular signaling and regulation of SNARE-mediated vesicle/granule exocytosis in numerous cell types. To investigate the potential requirement for caveolae in regulated insulin granule exocytosis from pancreatic β-cells, we studied interactions amongst the resident caveolar protein Caveolin-1 (Cav-1), Cdc42 and SNARE proteins and compared these structural interactions with their functional importance in glucose-stimulated insulin secretion. Subcellular fractionation analyses of MIN6 β-cells revealed Cav-1 distribution in both the plasma membrane and insulin granule fractions. Co-immunoprecipitation analyses revealed that Cav-1 associated with Cdc42-VAMP2 bound insulin granules in a glucose-dependent manner. Cav-1 interacted preferentially with GDP-bound GST-Cdc42 in pulldown analyses, and mutation/deletion of the Cav-1-scaffolding domain ablated this interaction. Sequence analysis of Cav-1 revealed a guanine nucleotide dissociation inhibitor (GDI) motif in the scaffolding domain and treatment of cells with GGTI-2147, a geranylgeranylation inhibitor resulted in a significant loss of Cdc42-Cav-1 binding. Functionally consistent with a role for Cav-1 as a Cdc42 GDI, siRNA-mediated depletion of endogenous Cav-1 increased basal insulin secretion by ~40%. Moreover, in Cav-1-depleted cells expression of the wild-type Cav-1 but not the

Cav-1 scaffolding domain mutant (F92A/V94A) compensated for the depletion and restored basal secretion. Taken together, these data support a model whereby Cav-1 interacts with the Cdc42-VAMP2 bound insulin granule complex and may contribute to the specific targeting of granules to active sites of exocytosis organized by caveolea and suggests that Cav-1 acts as a guanine nucleotide dissociation inhibitor to maintain basal secretion.

2637

**A Synapsin IIb Site 1 Phosphorylation Mutant Blocks Retention but not Insulin-Stimulated Release of GluT4 Vesicles**

J. M. Muretta, I. Romenskaia, C. C. Mastick; Biochemistry, University of Nevada, Reno, Reno, NV

Intracellular retention of GluT4 under basal conditions is a key mechanism for controlling glucose metabolism in adipocytes. However, the proteins regulating this process remain unknown. In neurons, the synapsin family of phosphoproteins are proposed to regulate synaptic vesicle (SV) traffic. Synapsins cluster SVs by binding to both vesicles and other synapsin molecules. Phosphorylation at serine 9/10 decreases their affinity for SVs, and is proposed to be a mechanism for stimulated release of the tethered vesicles. Synapsin 1 is expressed in liver epithelial cells and pancreatic β-cells, as well as in neurons. We found that adipocytes express synapsin IIb (protein and mRNA). Over-expression of phosphorylation mutant synapsin IIb (S10A) caused GluT4 vesicles to redistribute into perinuclear clusters. Unexpectedly, however, it also increased the amount of GluT4 at the plasma membrane (PM) 3-4 fold in basal cells. The surface content of the transferrin receptor (TfR) was unchanged. The GluT4 at the PM continually exchanged with the GluT4 in the perinuclear clusters under basal conditions. Furthermore, the GluT4 translocated out of the perinuclear clusters in response to insulin, with a further 2.5-fold increase at the PM. In control cells, insulin increased PM GluT4 6.5-fold. Insulin also increased PM TfR 2.5-fold, which was unaffected by S10A synapsin. After insulin stimulation, the levels of GluT4 at the PM, as well as its rates of exocytosis and endocytosis were the same in control cells and in cells over-expressing either WT or S10A synapsin. Additional data suggest that expression of S10A synapsin in adipocytes redistributes GluT4 from specialized storage compartments into the endosome where it co-localizes with TfR. We propose that synapsin may have a general function in recycling proteins from the endosome back into specialized compartments (i.e. GluT4-vesicles and SVs).

2638

**Small GTPase RNAi Screen of Insulin-mediated Insertion of GLUT4 into the Plasma Membrane of 3T3L1 Preadipocytes**

M. N. Teruel,[1] M. Kim,[1] A. Tengholm,[2] A. Salmeen,[1] T. Meyer[1]; [1]Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA, [2]Medical Cell Biology, Uppsala University, Uppsala, Sweden

The glucose transporter GLUT4 is a primary mediator of glucose uptake in muscle and fat tissue, and its insulin-triggered translocation from intracellular vesicles to the plasma membrane is likely relevant for attacking diseases such as diabetes and obesity. The molecular mechanisms that control GLUT4 trafficking still need to be elucidated. Using the Dicer method [1], we constructed a library of siRNA against 430 mouse small GTPases, GEFs, and GAPS. We co-transfected this library into 3T3L1 preadipoctes together with cDNA encoding YFP-GLUT4 with an HA-tag inserted in the first extracellular loop. We measured the amount of plasma membrane inserted GLUT4 before and after insulin stimulation by using a fluorescently-labelled antibody to the HA-tag [2]. Imaging was carried out in 96-well and 384-well format using an Axon ImageXpress automated epifluorescence imaging system. We have identified a number of small GTPases, GEFS, and GDIs with potential roles in different steps of GLUT4 transport, insertion, and recycling. [1] Myers et al. (2003) Nat Biotechnol. 21:324-8. [2] Quon et al. (1996) Biochem Biophys Res Commun. 226:587-94.

2639

**Examination of Complexin Function during SNARE-mediated Fusion In Vitro**

J. Schaub, J. Liao, J. A. McNew; Biochemistry and Cell Biology, Rice University, Houston, TX

The specific assembly of cognate SNARE proteins drives the fusion of synaptic vesicles with the presynaptic plasma membrane in neurotransmission. Syntaxin1A and SNAP25 form a t-SNARE complex localized to the plasma membrane that specifically pairs with the v-SNARE, VAMP2/synaptobrevin, located on the synaptic vesicle. We have been characterizing fusion driven by neuronal SNARE proteins and the accessory proteins involved in this process, specifically the neuronal regulatory proteins called complexins. The complexin isoforms are proteins found in the cytosol of neurons that bind to the assembled neuronal SNARE complex; however, the functional consequences of this association have yet to be determined. We utilize an in vitro fusion assay using reconstituted syntaxin1A/SNAP25 and VAMP2 to determine the effects of complexin on neuronal SNARE mediated fusion. When Drosophila melanogaster complexin, expressed in E. coli and purified by affinity chromatography, is added to fusion reactions containing mammalian neuronal SNAREs fusion activity is modestly, but reproducibly decreased. However, the addition of complexin to fusion reactions containing the homologous yeast plasma membrane SNARE proteins Sso1p/Sec9c;Snc2p does not inhibit fusion. Order of addition experiments suggest that complexin must be present as the v-t SNARE complex forms for inhibition to occur, in agreement with the observation that complexin binds primarily to the assembled ternary SNARE complex.

2640

**Assembly and Disassembly of the SNARE Complex Revealed at nm-Resolution**

A. M. Jeremic,[1] A. S. Quinn,[2] W. J. Cho,[1] D. J. Taatjes,[2] B. P. Jena[1]; [1]Department of Physiology, Wayne State University School of Medicine, Detroit, MI, [2]Department of Pathology and Cell Imaging Facility, University of Vermont, Burlington, VT

Target membrane proteins, SNAP-25 and syntaxin (t-SNAREs) and secretory vesicle-associated protein (v-SNARE), are part of the conserved protein complex involved in membrane fusion process. Results from our previous studies demonstrate that t-SNAREs and v-SNARE, when present in opposing bilayers, interact in a circular array to form conducting pores. The size of SNARE supramolecular complex formed is directly proportional to vesicle diameter. To further understand the dynamics and structural arrangement of SNARE complex following membrane fusion, the role of NSF (N-ethylmalemide-sensitive factor) in SNARE complex disassembly was explored. High-resolution imaging using atomic force microscopy (AFM) of single SNARE complexes formed on lipid membrane reveals distinct rings, each measuring a few nanometers in diameter. Addition of recombinant NSF and ATP to the incubation mixture results in rapid disassembly of the SNARE complexes. To further confirm this disassembly, immunochemical studies were performed. Immunoblot analysis demonstrates complete disassembly of SNARE complexes upon

addition of NSF and ATP. NSF alone, or NSF in the presense of the non-hydrolyzable ATP analog AMP-PNP, had no significant effect on the SNARE complex ($p>0.05$, n=4, Student's *t*-test), suggesting an enzymatic and energy-driven process. To further explore the effect of NSF on SNARE-mediated lipid-lipid interactions, real-time light scattering measurements of interacting t-SNARE and v-SNARE reconstituted vesicles in solution were performed. Similar to AFM and immuno blot studies, addition of NSF and ATP to the t/v-SNARE-vesicle mixture led to a rapid and significant increase in intensity of light scattering ($p<0.01$, n=3, Student's *t*-test), suggesting disassembly of the SNARE complex and dissociation of vesicles. These results demonstrate that NSF works downstream to the membrane fusion event, allowing rapid retrieval of secretory vesicles from the fusion site. Supported by NIH grants (BPJ).

2641

**Soluble Forms of SNAP-25 Reveal Specific SNARE Interactions and Dynamic SNARE Function in Secretion**

L. Wang, M. A. Bittner, M. W. Allersma, R. W. Holz; Pharmacology, University of Michigan, Ann Arbor, MI

Syntaxin, SNAP25, and synaptobrevin/VAMP form a core complex which plays an essential role in exocytosis. The steps in core complex formation are unknown. We investigated the ability of the two SNARE motifs of SNAP25 to bind VAMP2 and syntaxin1a in cells. The SNAP25 constructs were labeled with GFP variants and transiently expressed in Hek293T cells. In the absence of other transfected SNAREs, the N- and C-termini of SNAP25 (SN1 and SN2, respectively) had a diffuse cytosolic localization with little or no plasma membrane binding. Transiently expressed VAMP2 and syntaxin1a were localized to the plasma membrane as well as to other cellular membranes. In co-transfection experiments, SN1 colocalized with syntaxin on cellular membranes including the plasma membrane but not with VAMP. SN2 colocalized with either VAMP2 or syntaxin1a. When transiently expressed in chromaffin cells, SN2 was predominantly cytosolic, but with some binding to the plasma membrane in ~20% of the cells. In contrast, transiently expressed GFP-SNAP25 (full length) predominantly localized to the plasma membrane in chromaffin cells. Permeabilization resulted in the rapid loss (within seconds) of Cit-SN2 but not GFP-SNAP25. GFP-SNAP25 and Cit-SN2 (both with a mutation to render them resistant to botNT/E) protected nicotinic agonist-induced secretion from inhibition by the toxin. Thus, SN2 domains in both constructs can function in SNARE reactions leading to fusion. However, in permeabilized cells, GFP-SNAP25 but not Cit-SN2 rescued $Ca^{2+}$-dependent secretion, consistent with their abilities to remain in permeabilized cells. Conclusion: SNARE complex formation is a dynamic process, in which SN2 captures VAMP during formation of the core SNARE complex.

2642

**Cdc42 and Actin Control Polarized Expression of TI-VAMP-vesicles to Neuronal Growth Cones and Their Fusion with the Plasma Membrane**

T. Galli,[1] P. Alberts,[1] R. Rudge,[1] C. Gauthier-Rouvière,[2] T. Eirinopoulou[3]; [1]Cell Biology, Institut Jacques Monod, Paris, France, [2]Rho GTPases, Adhesion and Skeletal Muscle, CRBM-CNRS FRE 2593, Montpellier, France, [3]Inserm u536, Institut du Fer-à-Moulin, Paris, France

Tetanus neurotoxin Insensitive-Vesicle Associated Membrane Protein (TI-VAMP)-mediated fusion of intracellular vesicles with the plasma membrane is crucial for neurite outgrowth, a pathway not requiring tetanus neurotoxin-sensitive synaptobrevin-dependent exocytosis. Yet, it is not known how the TI-VAMP membrane trafficking pathway is regulated or how it is coordinated with the cytoskeletal dynamics that guide neurite outgrowth from the growth cone. Here, we demonstrate that TI-VAMP, but not synaptobrevin 2, concentrates in the peripheral, F-actin-rich region of the growth cones of hippocampal neurons in primary culture. Its accumulation correlates strictly with and depends upon the presence of F-actin as shown by the effect of actin sequestering drugs. Expression of a dominant-positive mutant of Cdc42, a key regulator of cell polarity, stimulates formation of F-actin- and TI-VAMP-rich filopodia outside the growth cone, an effect not seen when expressing Rac1 or RhoA dominant-positive mutants. Furthermore, we report that Cdc42 directly activates actin-dependent exocytosis of TI-VAMP-containing vesicles by imaging pHLuorin-tagged TI-VAMP. Collectively, our data indicate that Cdc42 and regulated assembly of the F-actin network control the accumulation and exocytosis of TI-VAMP-containing membrane vesicles in growth cones and suggest that membrane trafficking and actin remodeling are coordinated in neurite outgrowth.

2643

**Nucleotide-dependent Dissociation of Secretory Vesicles from the Plasma Membrane**

W. Cho, A. Jeremic, B. P. Jena; Department of Physiology, Wayne State University School of Medicine, Detroit, MI

Exocytosis is a fundamental cellular process that involves fusion of membrane-bounded secretory vesicle with fusion pores or porosomes and the expulsion of intravesicular contents. However, the fate of secretory vesicles following content discharge is less clear. In this study, we examined the dissociation of synaptic vesicles (SV) at the presynaptic membrane. To study SV turnover at the nerve terminals, high-resolution imaging of isolated synaptosomal membrane with docked synaptic vesicles were carried out using atomic force microscopy (AFM). Role of ATP-ase NSF, and the GTP-ase dynamin on SV-associated synaptosomal membrane was investigated. Exposure to ATP or GTP resulted in partial detachment of docked SV from the synaptosomal membrane. Simultaneous addition of ATP and GTP, however, led to significant release of the docked vesicles ($p<0.01$, n=4, Student's *t*-test). Addition of non-hydrolyzable ATP and GTP analogs had no significant effect on the dissociation of vesicles, suggesting an energy-dependent enzymatic-coupled process. Quantitative biochemical analysis of isolated synaptosomal membrane and synaptic vesicle fractions further confirmed detachment of docked SV upon addition of ATP and GTP. Similar results were also obtained using isolated plasma membranes of exocrine pancreas, suggesting a universal mechanism in the dissociation of secretory vesicles from the cell plasma membrane. Finally, immunoprecipitation studies using specific SNAP-25 antibody followed by western blot analysis, confirm the association of NSF and dynamin with SNAREs. Therefore, close proximity of NSF and dynamin to the SNARE complex along with their ability to efficiently dislodge secretory vesicle from fusion site suggests their role in cell secretion. Supported by NIH grants (BPJ).

2644

**Structural and Functional Insights into the Exocyst Protein Sec6p**

S. V. S. Mylavarapu, J. A. Saporita, M. Munson; Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA

In eukaryotic cells, small membrane-bound vesicles transport membrane and luminal cargo between intracellular organelles and to the cell surface.

Polarized exocytosis at specific sites on the plasma membrane requires a conserved, eight-subunit protein complex called the exocyst. Exocyst assembly at sites of secretion is essential for tethering the vesicle to the plasma membrane, prior to SNARE-mediated membrane fusion; it may also play a direct role in the SNARE complex regulation. The exocyst shares functional homology with tethering complexes that operate in other intracellular trafficking pathways. Moreover, its sequence conservation suggests a fundamentally important exocytic function in all eukaryotes. However, the structure, mechanisms of action, subunit organization and specific functions of the exocyst are unknown. Molecular characterization of the exocyst subunits individually and in combination is imperative for elucidation of the exocyst's function. Towards this end, we have biochemically and structurally characterized the yeast exocyst subunit Sec6p. Sec6p forms stable homodimers both *in vitro* and *in vivo*. Its N-terminal domain contains the dimerization region and interacts with the t-SNARE Sec9p. The C-terminal half of Sec6p is monomeric in solution and forms an independently folded structural domain (6CT). We have recently determined the crystal structure of this 6CT domain. 6CT is organized into three distinct helical bundles, with prominent solvent-exposed loops. Using structure-based sequence alignments, we identified a patch of highly conserved surface residues on one face of the structure. These residues likely mediate interactions with Sec6p's binding partners *in vivo*; studies are in progress to identify these partners. We have introduced site-specific mutants of these residues individually and combinatorially into yeast, to elucidate their physiological importance for Sec6p's function. The results from these studies will provide a framework for understanding the function of the exocyst at the molecular level.

2645

**Measurement of the Diameter of the Exocytotic Fusion Pore in the Pancreatic Acinar Cell**

J. A. Pickett, J. M. Edwardson; Department of Pharmacology, University of Cambridge, Cambridge, United Kingdom

In the pancreatic acinar cell, acetylcholine stimulates the fusion of zymogen granules with the apical plasma membrane, leading to the secretion of digestive enzymes. Exocytosis is inhibited by clostridial neurotoxins, highlighting the involvement of SNAREs in this process. Exocytotic membrane fusion in the acinar cell has several unusual characteristics, including the extraordinary stability (several minutes) of the fused granule, and the occurrence of compound exocytosis. We have used fluorescent membrane-impermeant dyes of various sizes to estimate the diameter of the exocytotic fusion pore. A lysine-fixable, 3,000 molecular weight (MW), Texas Red-dextran conjugate was used as an extracellular tracer for fused zymogen granules. Following acetylcholine stimulation, Texas Red-dextran labeled many exocytotic structures at the apical plasma membrane. The diameter of the open fusion pore was assessed by looking for co-localization of Texas Red-dextran (3,000 MW) with fluorescein-labeled dextran conjugates of various sizes: 3,000 MW (diameter ~2.5 nm), 70,000 MW (~11 nm), 500,000 MW (~28 nm), and 2,000,000 MW (~54 nm). All of the fluorescein-labelled dextran conjugates co-localized with Texas Red-dextran in the lumen of fused zymogen granules, but to varying extents. In particular, some, but not all, of the Texas Red-dextran positive granules were also positive for the 500,000 and 2,000,000 MW fluorescein dextrans. There was no difference between the labeling of primary or secondary fused zymogen granules. Our results show that the exocytotic fusion pore in the pancreatic acinar cell has a diameter exceeding 50 nm, suggesting that the pore radially expands to a stable state after the initial SNARE-dependent interaction between the two membranes.

2646

**Role of Calpain-10 as a Calcium Sensor in Exocytosis**

E. Aganna,[1] C. Marshall,[1] G. A. Hitman,[1] J. Burrin,[2] M. D. Turner[1]; [1]Diabetes and Metabolic Medicine, Queen Mary, University of London, London, United Kingdom, [2]Molecular Endocrinology, Queen Mary, University of London, London, United Kingdom

OBJECTIVE The molecular control of exocytosis by Ca2+-sensors in stimulus-secretion coupling is poorly defined. We present new data detailing calpain-10's contribution to these events. METHODS We examined the functional consequence of calpain-10 (CAPN10) overexpression and inhibition upon insulin secretion from INS-1 pancreatic beta-cells. We went on to compare CAPN10 expression profiles in INS-1 cells and two pituitary cell-lines, corticotropes (AtT20) and lactotropes (GH3), with their respective secretory profiles. RESULTS INS-1 basal secretion remains unaltered regardless of CAPN10 expression level. There is however a marked increase in stimulus-dependent regulated secretion that correlates with the extent of overexpression within physiological boundaries. Insulin secretory granule exocytosis is mediated by soluble N-ethyl maleimide sensitive fusion protein attachment receptor (SNARE) proteins. The t-SNAREs SNAP-25 and syntaxin 1 both co-immunoprecipitate with a ~ 54kDa membrane-associated calpain-10 isoform, indicating that calpain-10 is directly bound to the exocytotic granule fusion machinery. Furthermore there is a Ca2+-dependent partial proteolysis of SNAP-25 during exocytosis, which like regulated secretion itself is similarly sensitive to pharmacological calpain inhibition. Western analysis revealed a differential expression pattern between the 3 cell-lines, with the ~ 54 kDa calpain-10 isoform similarly expressed in the membrane fraction of both INS-1 and GH3 cells, but markedly reduced in AtT20 cells. This observation is consistent with secretion results, where calpain protease inhibitor similarly surpresses regulated secretion from both INS-1 and GH3 cells, but not AtT20 cells. CONCLUSION We hypothesise that calpain-10 is a Ca2+-sensor that mediates exocytosis in multiple endocrine cells. However it is not essential for exocytosis in all endocrine cells, thereby suggesting the participation of multiple Ca2+-sensors in stimulus-secretion coupling.

2647

**Calcium Signals Associated with 1,25D-Regulated Exocytosis in Osteoblasts**

P. Biswas, L. P. Zanello; Biochemistry, University of California-Riverside, Riverside, CA

The hormonally active form of vitamin D, 1α,25dihydroxy vitamin $D_3$ (1,25D), is considered a bone anabolic hormone. Osteoblasts produce and secrete a variety of bone matrix proteins. 1,25D increases bone matrix production by acting at the cell nucleus. In addition, 1,25D rapidly stimulates secretion of bone materials via nongenomic mechanisms initiated at the plasma membrane of bone cells. The present study investigates molecular pathways involved in 1,25D-regulated exocytosis in primary calvarial osteoblasts and osteosarcoma ROS 17/2.8 cells. Regulated type of secretion occurs as a rapid fusion of vesicles to the plasma membrane in response to a specific electrical or chemical stimulus. We found a rapid (seconds) exocytotic response triggered by nanomolar concentrations of 1,25D. Single exocytosis events were monitored in real time with confocal microscopy performed on live osteoblasts. Secretory granules were stained with 3 μM quinacrine. In addition, 1-100 nM 1,25D promoted a significant (2-fold) capacitance increase in single primary osteoblasts, as measured with patch-clamp techniques. Nifedipine (2 μM) and DIDS (200 μM) significantly reduced 1,25D-promoted capacitance changes, suggesting that this is associated with $Ca^{2+}$ and $Cl^-$ channel activation. Elevation

of cytoplasmic $Ca^{2+}$ by 10 nM 1,25D was measured with flow cytometry in large populations ($10^7$ cells) of ROS 17/2.8 cells labeled with the cell permeable $Ca^{2+}$-sensitive dye Fluo 3-AM. We conclude that a Ca signal is therefore likely to be the transducer of the hormonal stimulus into an electrical signal coupled to a rapid exocytotic response in osteoblasts. The anabolic effects of 1,25D can therefore be explained in part at the nongenomic level as a rapid promotion of exocytosis of bone materials by the hormone.

2648

**Rab27a and its Effector JFC1 Regulate Exocytosis in Human Neutrophils**

J. L. Johnson, D. B. Munafo, B. A. Ellis, S. D. Catz; Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA

Neutrophils play a central role in innate immunity by combating bacterial infections. Neutrophils kill microorganisms using microbicidal products that they release into the phagosome or the extracellular space. Mature neutrophils contain four types of exocytosable storage organelles: azurophilic granules [that contain myeloperoxidase (MPO)], specific granules, gelatinase granules and secretory vesicles. Since the uncontrolled release of the contents of these organelles is potentially harmful, granule exocytosis must be tightly regulated. However, the secretory machinery utilized by neutrophils is poorly characterized. The objective of this work is to characterize the role of Rab27a and JFC1 in the regulated secretion of neutrophils. Here, we show that JFC1 and Rab27a are highly expressed in human neutrophils. While Rab27a distribution is restricted to the membrane fraction after cell fractionation, JFC1 is distributed between the membrane fraction and the cytosol. Colocalization of endogenous JFC1 and Rab27a was observed by immunofluorescence analysis. Furthermore, when a two-layer Percoll gradient was used to separate the subpopulations of granules, JFC1 and Rab27a were mainly associated with the tertiary granules ($\gamma$ fraction) which are enriched in MMP-9 and cytochrome b558. A small proportion of the MPO-positive granules (~5%) were also detected in this fraction. The introduction of the plasma membrane binding domain (C2A domain) of JFC1 into permeabilized neutrophils significantly decreased exocytosis, however, neither Rab27a nor JFC1 integrated to the phagolysosome when neutrophils were exposed to opsonized particles, suggesting that the granules implicated in cargo release towards the surrounding milieu are molecularly and mechanistically different from those involved in their release towards the phagolysosome. Finally, the expression of JFC1 and Rab27a in HL-60 promyelocytic cells is dramatically increased when they are differentiated to neutrophil-like granulocytes, thus further supporting a role for these proteins in the secretory machinery of granulocytes.

2649

**Inhibition of the Vacuolar $H^+$-ATPase by Bafilomycin A1 Disrupts Targeting of Soluble and Membrane Proteins to Regulated Secretory Granules**

J. A. Sobota, B. A. Eipper, R. E. Mains; Neuroscience, University of Connecticut Health Center, Farmington, CT

The vacuolar $H^+$-ATPase (V-ATPase) is responsible for establishing and maintaining the pH gradient along the secretory and endocytic pathways. In the regulated secretory pathway, progressive acidification is thought to be important for proteolytic processing of prohormones, aggregation of soluble content, and secretory granule biogenesis. In order to investigate the role of the V-ATPase in targeting of soluble and membrane secretory proteins to granules, the pH gradient in AtT20 corticotrope cells expressing endogenous proopiomelanocortin was dissipated pharmacologically with the selective V-ATPase inhibitor bafilomycin A1. Alkalinization was verified by labeling cells with LysoSensor Green; fluorescence was abolished by 24 hour preincubation with nanomolar concentrations of bafilomycin. Following this treatment paradigm, localization of endogenous and GFP-tagged soluble secretory cargo was evaluated by confocal microscopy; both accumulate in large LAMP-1 positive structures with diameters up to 4μm. Granule membrane proteins similarly accumulate in these structures. The morphology of the trans-Golgi network remains intact. New protein synthesis is required for these effects, as concurrent treatment with bafilomycin and cycloheximide prevents formation of these structures and does not affect existing granules. Cells recover from drug treatment within 4 hours; the content of the enlarged structures is degraded, while newly synthesized secretory proteins are localized in granules. Secretory granules do not form during bafilomycin treatment, and the content of the LAMP-1 positive structures does not undergo regulated secretion. Constitutive secretion remains comparable to untreated cells. Alkalinization by chloroquine and ammonium chloride treatment does not induce these effects on secretory protein trafficking. Since these effects are specific to bafilomycin, we conclude that the V-ATPase, but not necessarily V-ATPase-mediated acidification, is required for proper targeting of newly synthesized soluble and membrane proteins to regulated secretory granules. Supported by DE017094-01, DK32948.

2650

**Modification of ATP Synthase Alpha Is Associated with Stimulated Acid Secretion by Gastric Glands**

L. Zhu,[1] S. Karvar,[1] J. Trinidad,[2] V. Lao,[1] A. L. Burlingame,[2] J. G. Forte[1]; [1]Molecular & Cell Biology, University of California, Berkeley, CA, [2]Department of Pharmaceutical Chemistry, University of California, San Francisco, CA

Drastic morphologic and functional changes occur when gastric parietal cells secrete HCl. When cells are stimulated with histamine, the apical membrane is expanded, recruiting most of the H,K-ATPase-rich tubulovesicles to the apical membrane where active secretion occurs. Another advantage of this material for studying proteomic changes is that the differences between resting and stimulated states can be maintained for extended periods. To get a global view of secretion-related changes, we prepared resting and stimulated gastric glands from rabbit. The quality of the material is ensured by assays of H,K-ATPase trafficking and acid secretion. Whole lysates from resting and stimulated glands were resolved by 2D electrophoresis. From Coomassie blue stained gels, the most pronounced difference between resting and stimulated samples was the pI shift of a 55kd protein. Although the percentage of the shift is different with different preparations, the 55kd protein repeatedly showed an acidic shift upon stimulation. In resting sample, much of the protein has an apparent pI of 8.3; in stimulated sample, the dominant mass was pI 7.7. We cut out the spots at pI 7.7, 8.0 and 8.3 for in-gel digestion and nano-LC-MSMS analysis. Results clearly identified these spots as ATP synthase alpha subunit. Western blot analysis confirmed the above results. Furthermore, ATP synthase shifted to a more basic value upon phosphatase treatment supporting phosphorylation modification. Western blot with anti-phospho-antibodies indicated that ATP synthase alpha is not Tyrosine phosphorylated, but possibly Serine-phosphorylated. We propose that there is a previously unrecognized mechanism of active regulation of ATP synthase, involving modification of the alpha subunit in a way that adds one or more negative charges. Active regulation of ATP synthase may be necessary for parietal cells to meet the energy demand of stimulated acid secretion.

2651

### Function of Parafusin and Its Modifications in Exocytosis

L. Liu, B. H. Satir; Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Parafusin (PFUS), a phosphoglycoprotein involved in $Ca^{2+}$-regulated exocytosis is regulated by phosphorylation and phosphoglucosylation. One of its orthologs *P*arafusin *R*elated *P*rotein 1 (PRP1) in *Toxoplasma gondii* is used here. To investigate the effects of protein modifications in exocytosis, an ortholog assay was developed. Purified recombinant His-tagged PRP1, labeled with $Alexa_{488}$, was electroporated into live *Paramecium* wt and localization observed by fluorescent microscopy. Like PFUS in wt cells, PRP1 localizes to docked secretory vesicles under the cell membrane. When stimulated, PRP1 becoming cytosolic. In a series of RNAi experiments we here test if knockdown of PFUS would result in an effect on exocytosis. A potential $exo^-$ phenotype could be derived from the knockdown in wt cells and reversal could be tested by electroporating PRP1 or PFUS into the $exo^-$ cells in complementation studies. The method chosen is RNA interference by feeding, as outlined in the paramecium database (http://paramecium.cgm.cnrs-gif.fr/RNAi/). We targeted three different regions of the PFUS sequence: the C-terminal (0.8 kb), the N-terminal (1.0 kb) and the entire open reading frame (1.8 kb). As a control we used the empty RNAi vector (LITMUS 28i). Exocytosis was scored after stimulation with trinitrophenol. Initial experiments using RNAi by feeding show that compared to controls after about 3-4 generations of growth roughly half the cells show only release from the anterior and posterior end of the cells (A/P phenotype) while the other half of the cell population is totally inhibited. Overall, exocytosis becomes inhibited with all three RNAi constructs compared to the control. Interestingly, inhibition progresses identically in a specific pattern with all three RNAi's.

## Caveolae (2652-2665)

2652

### Glycosphingolipid Structure and Plasma Membrane Microdomains: Determinants for Caveolar Endocytosis

R. D. Singh,[1] C. L. Wheatley,[1] E. L. Holicky,[1] Y. Liu,[2] D. L. Marks,[1] R. Bittman,[2] R. E. Pagano[1]; [1]Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, [2]Chemistry and Biochemistry, Queens College of CUNY, Flushing, NY

Caveolae are a subset of plasma membrane (PM) microdomains that are enriched in glycosphingolipids (GSLs) and cholesterol, and are associated with the protein caveolin-1. Caveolae are also involved in the clathrin-independent endocytosis of many membrane components (e.g., GSLs; β1-integrins) as well as some bacteria, viruses, and toxins. We have previously shown that fluorescent lactosylceramide (BODIPY-LacCer) and other GSL analogs are internalized almost exclusively *via* caveolae in human skin fibroblasts and other cell types. The molecular determinant for this selectivity is as yet unknown. We have systematically modified the structure of the GSL head group and sphingosine moiety and studied the endocytosis of these analogs. We found that the stereochemistry of the sphingosine base is critical for GSL uptake *via* caveolae. The two D-isomers of BODIPY-LacCer were taken up exclusively by caveolae while the two L-isomers underwent endocytosis *via* multiple mechanisms. Surprisingly, the non-natural L-*threo*-LacCer stereoisomer inhibited the caveolar internalization of other markers endocytosed by this mechanism. Finally, we utilized the monomer-excimer fluorescence of the BODIPY-analogs to investigate the formation of GSL microdomains at the surface of living cells. We show that only the D-LacCer analogs partition into plasma membrane microdomains induced either by CtxB clustering of endogenous $GM_1$ or by treatment with $C_8$-D-LacCer. Thus, partitioning of BODIPY-D-LacCer stereoisomers into specific microdomains correlated with their ability to be internalized *via* caveolae.

2654

### The PKC Binding Protein SDPR is Involved in Caveolae Invagination

H. K. Zajicek, K. McMahon, K. Luby-Phelps, Y. Ying, Y. Taketani, R. G. W. Anderson; Cell Biology, UT Southwestern, Dallas, TX

Loss of PKCα activity from cells blocks caveolae internalization. Previously, we showed that caveolae contain the PKCα binding protein SDPR (alias SDR, serum deprivation response protein). SDPR binds with high affinity (~100 nM) the regulator domain of PKCα, binding is calcium dependent and it appears to be necessary for localization of PKCα to caveolae. SDPR is also a PKCα substrate. SDPR is a 68 kDa protein that contains a PKCα binding site (aa 145-250), a putative leucine zipper (aa 52-100) and a PEST domain (aa 22-48). Here we report that SDPR appears to be required for caveolae invagination. Knockdown of SDPR RNA by RNA interference caused a loss of invaginated caveolae from the surface of human fibroblasts, as determined by quantitative electron microscopy (EM). Two regions of SDPR where identified that are necessary for its correct targeting to caveolae. The first is the leucine zipper. Immunofluorescence showed that deletion of this region or substitution of critical leucine residues with alanine blocked SDPR targeting to caveolae. The second is a twelve amino acids long (aa 150-161) potential phosphatidylserine (PS) binding site. A truncated SDPR (aa 1-168), which lacks the PKCα binding site, was correctly targeted to caveolae while a peptide that is missing the PS binding site (1-145) did not. To see if peptide 1-168 had a dominant negative affect on caveolae function, human fibroblasts where transfected with either the 1-168 or the 1-145 peptide. Quantitative EM showed a marked loss of invaginated caveolae in the cells expressing 1-168 compared to those expressing 1-145. We conclude that SDPR is a PKCα binding protein in caveolae that regulates caveolae internalization.

2655

### Caveolae-like Endocytosis in the Protozoan Parasite *Entamoeba histolytica*

N. M. León-Sicairos, M. Reyes-Lopez, M. de la Garza; Biologia Celular, CINVESTAV-IPN, México, Mexico

Caveolae are plasmatic membrane invaginations that contain the caveolin protein and cholesterol and are found in most cell types. Caveoale participate in endocytosis and delivered of proteins and lipids to diverse organelles in cells. *Entamoeba histolytica* is a pathogenic protozoan that causes amoebiasis, disease responsible of 100,000 deaths by year worldwide. Endocytosis is an important event in the pathogenicity of *E. histolytica*, however, endocytosis via caveolae in this parasite has not been described. The objective of this study was to determine if *E. histolytica* contains a caveolae-like endocytosis system. **Methods:** Confocal microscopy and flow cytometry were used to immunolocalize the caveolin-like protein and quantify cholesterol; the main markers of caveolae in cells. Also, the participation of caveolae-like in amoebic endocytosis was corroborated and the traffic of caveolae-like cargo in early and late endosomes and lysosomes. Ultracentrifugation was done to obtain caveolae, inmunoprecipitation and immunoblot assays were used to identify caveolin-like protein in amoebas. **Results.** A co-localization between caveolin

and cholesterol in the amoebic membrane was found. In endocytosis experiments using lactoferrin coupled to fluorescein, we found the co-localization of the three markers (caveolin, cholesterol and lactoferrin), inside amoebas, but when amoebas were pre-incubated with filipin, a drug that disrupts cholesterol and then caveolae structure and function, lactoferrin was not endocytosed. Using anti-bodies against early and late endosomal compartments and against lysosomes, we found that caveolin co-localized with these vesicles at different times. **Conclusions:** Vesicles located in the membrane of amoebas are enriched in cholesterol and in a caveolin-like protein of 21 kDa. These vesicles participated in *E. histolytica* endocytosis events, were filipin sensitive, and incorporated the cargo in the route of endosomes/lysosome. With these data we propose the existence of an alternative endocytosis system based in caveoale-like vesicles in *E. histolytica.*

2656

**Prostate Specific Membrane Antigen Associates with Caveolin-1 and Undergoes Caveolae-Dependent Internalization in Microvascular Endothelial Cells**

G. Anilkumar,[1] S. P. Barwe,[1] J. J. Christiansen,[1] S. A. Rajasekaran,[1] D. B. Kohn,[2] A. K. Rajasekaran[1]; [1]Pathology and Laboratory Medicine, University of California, Los Angeles, Los Angeles, CA, [2]Molecular Microbiology and Immunology, University of Southern California, Los Angeles, CA

Prostate Specific Membrane Antigen (PSMA) is a type II membrane protein expressed primarily in the secretory cells of the prostatic epithelium. PSMA expression is increased several fold in malignant disease, with the greatest levels observed in advanced tumors and androgen independent disease. PSMA is absent in most other tissues and cell types, including normal endothelial cells and pre-formed blood vessels, however, expression has been detected in the angiogenic sprouts of tumor-associated neovasculature for most solid cancers examined to date. This pattern of expression implies that PSMA may perform a functional role in angiogenesis and may offer a therapeutic target for the treatment of a broad spectrum of solid tumors. In an attempt to understand the significance of PSMA expression in the neovasculature, we have expressed PSMA in a well-characterized human microvascular endothelial cell line using retrovirus-mediated gene transfer. We have shown earlier that PSMA undergoes endocytosis via a clathrin-dependent mechanism in prostate epithelial cells. In this study, we demonstrate that PSMA associates with caveolin-1, a marker protein for caveolae; and that a substantial fraction of PSMA cosediments with the caveolar fraction, and undergoes internalization via a caveolae-dependent mechanism. The association between PSMA and caveolae in endothelial cells may provide important insight into PSMA function and ways to best exploit this protein for therapeutic benefit.

2657

**A Domain at N-Terminus of Caveolin-1 Controls Rear Polarization of the Molecule in Migrating Endothelial Cells**

X. Sun, D. C. Flynn, J. Liu; Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV

Caveolin-1, a caveolar membrane coating protein, plays an important role in signal transduction. Recently, we and others have shown that caveolin-1 polarizes at cell posterior in migrating endothelial cells and that caveolin-1 polarity is critical for endothelial cell migration. In this study, we generated a series of domain deletion mutants of caveolin-1 as GFP-fusion proteins and investigated domain or sequence that was required for caveolin-1 polarization in migrating cells. Our results demonstrate that $Cav_{1-178}$-GFP was polarized to the cell rear in migrating caveolin-1 deficient mouse embryonic fibroblasts (MEFs). In contrast, deletion of aa 1-60 prevented the molecule from polarization and, in turn, some of the mutant were located at the leading edge, suggesting that aa 1-60 were essential for caveolin-1 polarization. Interestingly, deletion of aa 1-60 did not prevent caveolin-1 polarization in HUVECs as well as Cav-1 wild-type MEFs, suggesting that $Cav_{61-178}$-GFP mutant might be interacting with endogenous caveolin-1 during polarization. Consistent with this, $Cav_{61-178}$-GFP and $Cav_{61-178}$-Flag were co-immunoprecipitated with endogenous caveolin-1. Furthermore, $Cav_{61-178}$ was incorporated into light membrane domains and mediated caveolae formation. Our results indicate that aa 1-60 in caveolin-1 were essential for its polarization in migrating cells.

2658

**Recruitment of Caveolin-1 to Focal Adhesions Initiates Focal Adhesion Disassembly at the Trailing Edge of Migrating Cells**

A. R. Beardsley,[1] X. Sun,[1] L. M. Millecchia,[2] M. D. Schaller,[3] K. M. Bailey,[1] J. Liu[1]; [1]Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV, [2]Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health, Morgantown, WV, [3]Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC

Cells migrating on a planar surface develop a characteristic front-rear structural asymmetry with coordinate functions. Actin nucleation and lamellipod protrusion takes place at the cell leading edge while de-adhesion and retraction occur at cell posterior. De-adhesion is mediated by the weakening and loss of focal adhesions, but the molecular events precipitating focal adhesion disassembly are largely unknown. Recent studies have demonstrated the polarization of caveolin-1 to the rear of migrating endothelial cells. To better understand the function of polarized caveolin, we used live-cell imaging to observe human endothelial cells expressing caveolin-1 fused to green fluorescent protein (GFP). Time-lapse confocal and differential interference contrast imaging revealed concentration of polarized caveolin into trailing fibers that formed as the cells advanced. Caveolin-1 recruitment was followed immediately by retraction of the trailing fibers into the cell body. Further imaging of cells co-expressing caveolin-1 fused with red fluorescent protein and paxillin-GFP demonstrated that caveolin-1 was recruited specifically to focal adhesions, leading to sliding and disassembly of the adhesions. Furthermore, caveolin-1 ablation caused a defect in cell de-adhesion, as evidenced by an increase in length and persistence of trailing edges in migrating caveolin-1 null fibroblasts relative to wild-type cells. Altogether, the data suggest that polarized caveolin-1 functions to initiate de-adhesion at the cell rear during migration.

2659

**Inhibition of PI 3-kinase and Myosin II Blocks Caveolin-1 Polarity in Migrating Endothelial Cells**

W. Fu, J. Liu; Physiology and Pharmacology, West Virginia University, Morgantown, WV

Endothelial cell migration is a key step towards angiogenesis, a process that is required in a variety of physiological and pathological conditions, such as embryonic development, wound healing, tissue regeneration and tumor growth and metastasis. The precise mechanism of chemoattractant-induced directional movement of endothelial cells is largely unknown. Recently, we have demonstrated that caveolin-1 polarized at the rear of migrating endothelial cells and that loss of caveolin-1 polarity impeded endothelial cell directional movement. To examine signal pathways that

may affect caveolin-1 polarity, we treated human endothelial cells with specific inhibitors for PI 3-kinase such as wortmannin and LY294002. Treatment with wortmannin caused a dose-dependent inhibition of caveolin-1 polarization. At the concentration of 50nM, wortmannin inhibited the number of caveolin-1 polarized cells by 50%. To assess whether myosin II was involved in caveolin-1 polarization, we treated endothelial cells with a specific inhibitor for myosin II, blebbistatin, and show that blebbistatin inhibited the number of caveolin-1 polarized cells by more than 30%. Our results are consistent with the role of PI 3-kinase/Akt signaling in the control of cell polarity, myosin II assembly and chemotaxis.

2660

**Cav-1 Downregulates Cox-2 Expression Level Through Proteasome Degradation Pathway**

S. Chen,[1,2] S. Shyue[2]; [1]National Defense Medical Center, Taipei, Taiwan Republic of China, [2]Institute of Biomedical Science, Taipei, Taiwan Republic of China

We have previously shown that interleukin-1β (IL-1β) induced cyclooxygenase-2 (COX-2) interacted and colocalized with caveolin-1 (Cav-1), the major component of caveolae. Numerous studies suggest that both COX-2 and Cav-1 play important roles in many diseases including tumorigenesis and maintenance of cardiovascular homeostasis. Although the degradation of COX-2 is important in regulation of its enzymatic activity and associates with many diseases status, study related to COX-2 degradation as well as its post-translational regulation remain mostly unknown. In this study, we investigated the effect of Cav-1 related to COX-2 expression and degradation. Although constitutive expressed COX-2 was suggested in HT-29 cells, low level of COX-2 was detected without induction. COX-2 can be induced by IL-1β to high level at 9 to 12 h and declined thereafter. In mock-transfected and control cells COX-2 levels and activities were increased with IL-1β induction. However, overexpression of Cav-1 significantly attenuated COX-2 expression and its activity. Since MG-132, a proteasome inhibitor, reversed the suppression effect of Cav-1, COX-2 degradation is mediated via the proteasome degradation pathway. Besides, COX-2 mRNA was not altered in Cav-1 overexpressed HT29 cells assayed by RT-PCR. These results suggest that Cav-1 plays an important role in regulating COX-2 expression by enhancing COX-2 degradation through the proteasome degradation pathway.

2661

**Roles of Caveolin-1 and ABCA1 on Cholesterol Efflux in Rat Aortic Endothelial Cells**

Y. C. Lin,[1] W. W. Lin,[2] V. C. Yang[1]; [1]Department of Life Science, Tunghai University, Taiwan, Taichung, Taiwan Republic of China, [2]Division of Cardiology, Taichung Veterans General Hospital, Taiwan, Taichung, Taiwan Republic of China

Atherosclerosis is a most common cause of death in the world. Reducing accumulation of cholesterol in the arterial walls can prevent development of atherosclerosis. Caveolae, a small membrane invaginations on the cell surface, and ABCA1, a transmembrane protein, have both been implicated in the HDL-mediated cellular cholesterol efflux. However, the underlying mechanism in cholesterol efflux is still unknown. Our previous study demonstrates that ABCA1 may act as a structural platform between HDL and caveolin-1, the major structural component of caveolae, on the cell surface during cellular cholesterol efflux. In this study, the cholesterol efflux was evaluated by up-regulation and down-regulation of caveolin-1 gene expression in rat aortic endothelial cells (RAECs). For the up-regulation of caveolin-1 in ECs, pCAV-cDNA expression vectors were constructed and transfected into the cells with lipofectamine. The down-regulation of caveolin-1 was carried out by siRNA. The cholesterol efflux was determined in the [$^3$H]cholesterol-loaded ECs after the aforementioned treatments. Our results indicated that the cholesterol efflux increased about 32% in the caveolin-1 up-regulated cells and decreased about 30% in the siRNA-treated cells as compared with controls. We suggest that caveolin-1 is a positive regulator of cholesterol efflux in RAECs. We also found that the probucol, an inhibitor of ABCA1-mediated lipid efflux, could partially inhibit the cholesterol efflux in the caveolin-1 up-regulated cells. Furthermore, the caveolin-1 siRNA-treated cells down-regulated the expression of ABCA1 and disturbed the colocalization of ABCA1 and HDL. According to these results, we suggest that caveolin-1 and caveolae are necessary for the interaction between ABCA1 and HDL during cholesterol efflux.

2662

**Expression of Caveolin-1 in Human T-cells**

H. M. Maldonado,[1] D. Sosa,[1] A. Rivera,[2] W. I. Silva,[3] G. Velazquez[3]; [1]Pharmacology, Universidad Central del Caribe, Bayamon, PR, [2]Physiology, Universidad Central del Caribe, Bayamon, PR, [3]Physiology, University of Puerto Rico, Medical Science Campus, San Juan, PR

Caveolin-1 (cav-1) has been recognized as a scaffolding protein that concentrates, organizes, and in some instances modulates the function of many membrane associated proteins including several signal transduction elements. Although its presence has been established in most cells, expression of this protein in T-cells remains controversial. In a recent report, a key role for cav-1 in T-cell HIV infection has been suggested (Hovanessian et al., Immunity 21:617, 2004). Moreover, several of the signal transduction pathways implicated in T-cell activation have also been associated with cav-1 in many other cells. In this report we present conclusive evidence of the presence of cav-1 in both the leukemic cell line Jurkat as well as human $CD4^+$, $CD8^+$, and $CD19^+$ lymphocytes. Expression of cav-1 mRNA is demonstrated with real time RT-PCR, while expression of the protein is detected with Western Blot and flow cytometry. Furthermore we have also found the typical methyl-β-cyclodextrin sensitive association of this protein with light density detergent resistant membrane fractions (lipid rafts). Immunofluorescent localization of this protein by confocal microscopy revealed the classical granular distribution pattern observed in many other cells. Finally, T-cell activation resulted in a significant up-regulation of cav-1 and a change in distribution pattern of both cav-1 and CD3 in a capping pattern. Supported by grants S06-GM50695, S06-GM08224, and 2G12RR03035 awarded to HMM, WIS, and AR respectively.

2663

**Mechanism of SRBC Targeting to Caveolae**

K. McMahon,[1] H. Zajicek,[1] W. Li,[1] K. Luby-Phelps,[1] M. Peyton,[2] J. Minna,[2] R. G. W. Anderson[1]; [1]Cell Biology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, [2]Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, Dallas, TX

Detergent-free caveolae proteomics identified a small family of C-kinase interacting proteins that are enriched in caveolae. This family includes SDR, SRBC and PTRF. All three members have a similar domain organization consisting of an N-terminal leucine zipper and a PKC binding site. We have focused on SRBC, which was originally isolated as an interacting partner and substrate for PKC delta and has since been shown to

interact with BRAC1. SRBC is downregulated in several lung and breast cancers, suggesting that it may act as a tumor suppressor. Here we demonstrate that SRBC is a caveolar protein that has a high coefficient of correlation with caveolin-1 by immunofluorescence. Tissue distribution of SRBC suggests it is ubiquitously expressed. The SRBC gene produces by alternative splicing, six different protein isoforms. SRBC depends on caveolin-1 for localization to caveolae. When SRBC-GFP alone is introduced into caveolin-1 knockout cells, SRBC-GFP exhibits a diffuse cellular localization. Expressing caveolin-1-Myc together with SRBC-GFP in these cells restores the co-localization with SRBC at the cell surface. Knockdown of caveolin-1 using RNAi causes the loss of SRBC protein suggesting that SRBC may also be stabilized by caveolin-1. Deletion of the leucine zipper domain shifts the localization of SRBC to the cytosol, demonstrating that this domain contains information necessary for targeting SRBC to caveolae. When we monitored SRBC-GFP movement at the cell surface by TIRF, we observed a "kiss and run" like behavior similar to caveolin-1-GFP. Therefore, SRBC appears to be a cytoplasmic signaling protein that is targeted exclusively to caveolae.

2664

### Organelle Type (Na+, K+)/H+ Exchanger NHE7 Partly Associates with Caveolae

P. J. C. Lin, M. Numata; Biochemistry and Molecular Biology, The University of British Columbia, Vancouver, BC, Canada

Acidic luminal pH in intracellular organelles along secretory and endocytic pathways plays crucial roles in vesicular trafficking and signal transduction. Mammalian ($Na^+$, $K^+$)/$H^+$ exchanger NHE7 predominantly localizes to acidic organelles, such as the *trans*-Golgi network (TGN) and endosomes at steady state. Although NHE7 is proposed to be a key regulator of organelle pH and other monovalent cation homeostasis, little is known about its biological function and the regulation mechanisms. Here, we show that NHE7 partly associates with caveolae and heterologous expression of previously reported caveolin-1 dominant negative constructs diminishes the caveolae accumulation of NHE7. We also report a novel dominant negative caveolin-1 mutant that disrupts the NHE7-caveolae association. We found that NHE7 is N-glycosylated in CHO cells and the N-glycosylated form accumulates preferentially to the caveolae fractions. However, inhibition of N-glycosylation by tunicamycin did not dissociate NHE7 from the caveolae fractions. GST pull-down experiments revealed that the cytosolic C-terminus of NHE7 directly interacts with the C-terminal membrane association domain (C-MAD) of caveolin-1. Most of the previously identified caveolin binding proteins bind to the N-terminal caveolae scaffolding domain (CSD). Thus, NHE7-caveolin interaction represents a novel module of caveolin-binding. Potential implications of caveolae/caveolins in NHE7 transporter functions and targeting will be discussed.

2665

### Growth Hormone Receptor Determinants for Lipid Rafts Localization

N. Yang,[1] X. Wang,[2] J. Jiang,[2] S. J. Frank[2]; [1]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [2]Department of Medicine, University of Alabama at Birmingham, Birmingham, AL

The growth hormone receptor (GHR) is a cell surface receptor that mediates the somatogenic and metabolic effects of GH. GHR signaling is transduced via the receptor-associated cytoplasmic tyrosine kinase, JAK2. The major intracellular signaling systems activated by JAK2 in response to GH include the signal transducer and activator of transcription (STAT) 5 and extracellular signal-regulated kinase (ERK)-1 and -2 pathways. Our previous study suggests that GHR is highly concentrated in the cholesterol-rich microdomains (caveolae and lipid rafts) of the plasma membrane and such subcellular localization of GHR plays important roles in GH signaling. In the current study, we investigate the determinant(s) for targeting GHR to caveolae/lipid rafts. By comparing GHR-expressing JAK2-deficient human fibrosarcoma cells (γ2A-GHR) with those reconstituted with JAK2 (γ2A-GHR-JAK2), we found JAK2 expression had no effect on GHR caveolar localization. We further looked for region(s) required for caveolar localization within GHR itself by reconstituting GHR mutants into GHR-deficient γ2A-JAK2 cells. $GHR_{1\text{-}274\text{-}Myc\text{-}His,}$ which includes extracellular domain (ECD), transmembrane domain (TMD), and four cytoplasmic domain residues with C-terminal Myc and His tags, was similarly concentrated in the caveolae/rafts as wt GHR was. However, $GHR_{239\text{-}620,}$ which lacks most of the ECD but includes the stem region, was markedly less concentrated in the caveolar fraction (about 2.6-fold) than wt GHR and $GHR_{1\text{-}274\text{-}Myc\text{-}His}$ (more than 8.6-fold for each). In addition, two GHR mutants with deletions within stem region, $GHR_{\Delta 237\text{-}243}$ and $GHR_{\Delta 242\text{-}244,}$ also showed similar caveolar enrichment as wt GHR does. These results suggest that GHR caveolar targeting information may reside mainly in the ECD with contribution from the TMD, and the stem region is probably not important in targeting GHR to caveolae/lipid rafts.

## Trafficking in Polarized Cells (2666-2682)

2666

### Rab11a Is Required for Canalicular Biogenesis in WIF-B9 Hepatic Cells

Y. Wakabayashi,[1] P. Dutt,[2] J. Lippincott-Schwartz,[1] I. M. Arias[1]; [1]Cell Bioligy and Metabolism, NICHD, NIH, Bethesda, MD, [2]NIAID, NIH, Bethesda, MD

Hepatocytes polarize by forming functionally distinct sinusoidal (basolateral) and canalicular (apical) plasma membrane domains. Two distinct routes are utilized for delivery of membrane proteins to the canaliculus. Proteins having GPI anchors or single transmembrane domains are targeted to thesinusoidal plasma membrane from where they transcytose to the canalicular domain. In contrast, apical ATP-binding-casette (ABC) transporters, which are required for energy-dependent biliary secretion of bile acids (ABCB11), phospholipids (ABCB4) and nonbile acid organic anions (ABCC2), lack initial residence in the basolateral plasma membrane and traffic directly from Golgi to the canalicular membrane. While investigating mechanisms of apical targeting in WIF-B9 cells, a polarized hepatic epithelial cell line, we observed that rab11a is required for canalicular biogenesis. Knockdown of rab11a or overexpression of rab11a-GDP locked form prevented canalicular biogenesis as did overexpression of myosin Vb motorless tail domain. In nonpolarized WIF-B9 cells, apical ABC transporters colocalized with transcytotic membrane proteins in rab11a-containing endosomes and, unlike the transcytotic markers, did not distribute to the plasma membrane. We propose that polarization of hepatocytes (ie, canalicular biogenesis) requires recruitment of rab11a and myosin Vb to intracellular membranes which contain apical ABC transporters and transcytotic markers permitting their targeting to the plasma membrane. In this model, polarization is initiated upon delivery of rab11a-myosin Vb-containing membranes to the surface which causes plasma membrane at the site of delivery to differentiatate into apical domain (bile canaliculus).

2667

**MAL2 Is a Possible Regulator of Polarized Apical Protein Sorting in Nonpolarized Hepatic Cells**

S. Ramnarayanan, P. L. Tuma; Biology, The Catholic University of America, Washington, DC

We have been investigating polarized membrane trafficking in nonpolarized cells. One current hypothesis proposes that nonpolarized cells are polarized cells waiting to happen; they have the requisite machinery, and thus capacity, for polarized plasma membrane (PM) delivery, but lack the spatial organization of distinct membrane targets. Consistent with this hypothesis, we found that would-be apical proteins were present at the PM in nonpolarized hepatic cells, but were also specifically sorted to a novel compartment containing only other apical proteins and that this sorting was cholesterol and glycosphingolipid dependent. We propose that this sorting from the PM to the novel compartment is the nonpolarized cell's version of transcytosis. To test this hypothesis, we have been examining MAL2, a raft-associated proteolipid and proposed regulator of transcytosis, in nonpolarized, hepatic Fao cells. MAL2 colocalized with apical proteins in the novel compartment, but was absent from the PM. In cells treated with nocodazole, the apical compartment was dispersed and MAL2 redistributed to the PM suggesting that MAL2 may recycle between the apical compartment and the PM, an itinerary shared by apical residents. Treatment with methyl-β-cyclodextrin also led to the PM redistribution of MAL2 further suggesting this recycling is cholesterol-dependent. Actin disruption with latrunculin B led to the intracellular accumulation of MAL2, indicating its itinerary is also dependent on an intact actin network. When overexpressed, MAL2 was present in the apical compartment, but was also present in large intracellular puncta that did not contain apical proteins. MAL2 overexpression also altered the distribution of the apical PM proteins suggesting MAL2 regulates delivery of apical proteins from the PM to the apical compartment. Together, these results suggest that the transcytotic pathway is conserved in nonpolarized Fao cells.

2668

**Membrane Microdomains in Hepatocytes: Potential Target Areas for Proteins Involved in Canalicular Bile Secretion**

J. R. Jefferson,[1] A. Mazzone,[2] P. Tietz,[2] R. E. Pagano,[2] N. F. LaRusso[2]; [1]Chemistry, Luther College, Decorah, IA, [2]Thoracic Diseases Research Unit, Mayo Clinic and Foundation, Rochester, MN

Formation of hepatic bile requires that large volumes of water be rapidly transported across liver epithelia, including hepatocytes. Aquaporins (AQPs), are a family of water channel proteins involved in bile secretion. Three AQPs are expressed in rat hepatocytes: AQP8, AQP9, and AQP0. Upon exposure of hepatocyte couplets to a choleretic agonist (dibutyryl cyclic AMP), AQP8, the most abundant AQP in hepatocytes, is redistributed to the canalicular plasma membrane, while the subcellular distributions of AQP0 and 9 are unaffected. This study investigated the hypothesis that canalicular bile secretion involves the regulated trafficking of vesicles to and from lipid-enriched microdomains within the canalicular plasma membrane. Toward this goal, microdomains were isolated by isopycnic centrifugation, followed by sonication in Triton and centrifugation on a sucrose gradient. A Triton-insoluble, sphingolipid-enriched microdomain fraction at the 5/30% sucrose interface was recovered and found to be enriched in alkaline phosphatase (microdomain-positive marker) and devoid of amino peptidase-N (microdomain-negative marker). Microdomains prepared from both apical and basolateral fractions tested positive for caveolin-1 (a "raft" associated protein) and negative for clathrin (a "raft" negative protein). The apical fraction was enriched in alkaline phosphatase while the basolateral was enriched in Na/K ATPase. Lipid analysis of these isolated microdomains indicated that both apical and basolateral fractions were highly enriched in cholesterol and sphingolipids. Exposure of isolated hepatocytes to glucagon, a choleretic agonist, significantly increased the expression of AQP8 associated with the apical microdomain fractions but had no effect on AQP9 expression in the basolateral microdomain fraction. The data suggest that microdomains represent targets for the exocytic insertion and retrieval of "flux proteins", including AQPs, involved in canalicular bile secretion. This work was supported by the National Institutes of Health Grant DK 24031 and by the Mayo Foundation.

2669

**Mapping Out the Itinerary of the Proteolipid, MAL2, in Polarized Hepatic Cells**

J. G. In, C. A. Cheng, P. L. Tuma; Biology, The Catholic University of America, Washington, DC

Our long term goal is to understand how hepatic cells establish and maintain their polarity. Our focus is to identify regulators of apical plasma membrane (PM) delivery. Unlike simple epithelial cells that directly target proteins from the TGN to the apical PM, hepatocytes use an indirect pathway: proteins are first delivered to the basolateral domain and then selectively internalized and transcytosed to the apical surface. MAL2 has been identified as a regulator of indirect apical delivery and is proposed to function between the basolateral early endosome (BEE) and the sub-apical compartment (SAC). In polarized WIF-B cells, we localized MAL2 to the apical PM, which presented us with a paradox. How can MAL2 function at the BEE when it is present at the apical pole? Upon closer examination, we noticed that MAL2 was also present in sub-apical structures. After incubation at 18°C, MAL2 accumulated in these structures while staining decreased reciprocally from the apical PM, suggesting MAL2 was present in the SAC. MAL2 colocalized with transcytosing apical residents and basolaterally internalized endolyn-78 in sub-apical structures, confirming the presence of MAL2 in the SAC. In cells treated with methyl-β-cyclodextrin or nocodazole, MAL2 redistributed to the basolateral PM indicating this domain as a step in MAL2's itinerary. To determine if MAL2 traversed BEEs, we double labeled MAL2 with internalized transcytosing apical residents or endolyn-78 in cells treated with nocodazole. This treatment inhibits delivery from the BEE to the SAC, leading to the endosomal accumulation of internalized proteins. MAL2 partially co-localized with the accumulated proteins in treated cells indicating that its itinerary includes BEEs. Together, these data indicate that MAL2 is an itinerant protein that traverses transcytotic intermediates consistent with its proposed role in regulating delivery between the BEE and SAC.

2670

**Molecular and Cellular Determinants of the Asymmetric Distribution of the Low-Density Lipoprotein Related Protein, LRP, in Polarized Cells**

M. Donoso,[1] A. Cáceres,[2] C. Retamal,[1] G. Bu,[3] V. Malhotra,[4] A. Gonzalez,[1] J. Cancino,[1] M. P. Marzolo[1]; [1]Depto.Biología Celular y Molecular, Pontificia Universidad Católica de Chile, Santiago, Chile, [2]Instituto de Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET), Córdoba, Argentina, [3]Department of Pediatrics, Washington University School of Medicine, St. Louis, MO, [4]Cell and Developmental Biology Department, University of California San Diego, San Diego, CA

LRP is expressed in several cell types, including polarized cells, neurons and epithelial cells. We have shown that the basolateral distribution of

LRP is determined by two cytosolic tyrosine residues and by the adaptor complex AP-1B (1). Here, we have extended our studies towards the understanding of the molecular and cellular elements that govern the polarized distribution of LRP in two epithelial cell lines and in hippocampal neurons. Besides the role of the tyrosines residues of the first NPxY and the shared NPxY/Yxxϕ motifs, a distal dileucine motif had also a role in the polarized distribution of LRP. All the mutants that changed the receptor´s distribution from basolateral to apical or non-polarized distribution of LRP in epithelial cells, correspondingly changed its distribution from a somatodendritic to axonal or non-polarized in hippocampal neurons. Interestingly, the mutation of the first NPxY motif by replacing by alanine, either the N or the Y, had a completely different effect in the distribution of the receptor, being the N26A mutant completely intracellular, co-localizing with EEA1, in contrast with the apical/axonal distribution of the and Y29A mutant. This motif is the same one recognized by SNX17 (2). By microinjection experiments, we determined that the mutant Y29A traffics directly to the apical domain, while the mutant N26A goes to the basolateral pole, is endocytosed, but unable to recycle back to the cell surface. Finally, we showed that the basolateral distribution of LRP depends on the activity of protein kinase D2, since the receptor´s distribution was reverted to the apical membrane upon expression of a kinase dead mutant, which would affect the LRP exit from the TGN (FONDECYT 1020746; FIRCA TW006456; FONDAP 13980001 ; MIFAB)

2671

**Distinct BFA-Induced Tubulation of Apical and Basolateral Membranes at the TGN**

K. N. Kitt, J. M. Wilson; Cell Biology and Anatomy, University of Arizona, Tucson, AZ

The establishment and maintenance of cell polarity is an active process that requires specific sorting and targeting to the apical and basolateral plasma membrane domains of the cell. Our lab has identified the small GTPase Rab14 as part of the regulatory machinery for apical targeting in polarized cells. We have shown that Rab14 is associated with the *trans*-Golgi network (TGN) and apical endosomes. In addition, expression of Rab14 S25N (inactive mutant) causes the TGN to expand and missort apical cargo. This led us to implicate Rab14 in the process of budding of vesicles from the TGN. However, the role of Rab14 in this process is not understood. To address the possible role of Rab14 in budding from the TGN we utilized Brefeldin-A (BFA). As shown by others, BFA causes Golgi compartments to redistribute into the ER and induces tubulation of the *trans*-Golgi network (TGN), endosomal, and lysosomal compartments. We found that addition of BFA to NRK cells causes extensive tubulation of Rab14 wt and Rab14 Q70L (active mutant) compartments and some tubulation of the portion of the TGN that contains the basolaterally targeted protein TGN38. Importantly, the BFA-induced tubules contain only TGN38 or Rab14. However, no tubulation of Rab14 occurs in NRK cells expressing Rab14 S25N, but tubulation of TGN38 still occurs. These data show that BFA differentially affects domains of the TGN containing apical and basolateral proteins and suggests that inactive Rab14 cannot associate with cytoskeletal elements to allow tubulation. We are currently investigating the possible association of Rab14 with microtubules and/or motor proteins.

2672

**Significance of Cholesterol in IgA-Receptor Transcytosis**

J. P. Leyt, S. Cohen, B. Aroeti; Hebrew University of Jerusalem, Jerusalem, Israel

Cholesterol enriched lipid rafts serve as platforms for a broad range of cellular activities. They typically resist solubilization in Triton X-100 in the cold and buoy in floatation gradients as detergent resistant membranes (DRMs). OBJECTIVE: To explore the significance of cholesterol-rich domains in the post-endocytic trafficking of the dimeric IgA (dIgA)-receptor (dIgA-R) and human transferrin receptor (hTfnR) expressed in polarized MDCK epithelial cells. RESULTS: In steady state, the dIgA-R is largely excluded from DRMs. However, upon 1 min exposure to the ligand at the basolateral surface, a fraction of the receptor was incorporated into DRMs, suggesting that dIgA binding stimulates the incorporation of its receptor into rafts in the vicinity of the basolateral surface. A fraction of the hTfnR was also associated with DRMs. However, hTfn binding had no effect on receptor association with that fraction. Cell surface biotinylation combined with floatation analyses revealed that hTfnR in DRMs originated at the basolateral plasma membrane, diminishing upon receptor internalization into endosomes. Cholesterol depletion of MDCK cells (with mβCD) before ligand internalization resulted in depolarized post-endocytic trafficking of both, dIgA and hTfn. Constitutive transcytosis of the empty dIgA-R was unaffected by that treatment. Interestingly, cholesterol depletion after ligand endocytosis selectively stimulated the kinetics of dIgA transcytosis. Cholesterol enrichment caused selective inhibition of dIgA transcytosis. A brief uptake of dIgA resulted in activation of Rac-1, whereas prolonged chase of the ligand diminished Rac-1 expression. Cholesterol enrichment counteracted those effects. CONCLUSIONS: Our data suggest that cholesterol-rich microdomains at the basolateral surface of MDCK cells are involved in polarized post-endocytic sorting events. Cholesterol in the basolateral surface and endosomes plays a role in polarized sorting of transcytotic dIgA, possibly via a mechanism that involves the activation of Rho and Rac- dependent pathways that control local actin and microtubule stabilization.

2673

**Beta-2-spectrin Localizes to a Novel Microtubule Dependent Intracellular Tubulovesicular Compartment Distinct from the Golgi that is Enhanced by Brefeldin a in Polarized Epithelial Cells**

K. Kizhatil, K. M. Abdi, V. Bennett; Cell Biology, Duke University, Durham, NC

Recently we have shown that beta-spectrin collaborates with the ankyrin-G in lateral membrane formation and that beta-2-spectrin is required for lateral membrane formation in cultured epithelial cells as well as the early mouse embryo[1]. Here we have analyzed beta-2-spectrin localization in human bronchial epithelial cells. We used confocal microscopy coupled with three dimensionl rendering to obtain high resolution light images of the localization of beta-2-spectrin in bronchial epithelial cells. Ankyrin-G and beta-2-spectrin both localize to the lateral membrane in bronchial epithelial cells. However, beta-2-spectrin also is localized independent of ankyrin-G, E-cadherin, and the Na/K ATPase in an intracellular tubulovesicular compartment. This compartment is juxtaposed to the cytoplasmic face of the lateral membrane as well as in an intracellular distribution. Treatment of cells with BFA markedly increased intracellular beta-2-spectrin-labeled structures. Beta-2-spectrin does not co-localize with markers for conventional Golgi, ER, and endosomes. Adducin, a component of the spectrin network, also localizes to intracellular tubulovesicular structures similar to beta-2-spectrin. Disruption of microtubules disperses the beta-2-spectrin compartment and also reduces levels of beta-2-spectrin on the lateral membrane. This study reports discovery of a novel beta-2-spectrin-containing tubulovesicular compartment distinct from Golgi that is enhanced by BFA and requires microtubules. Considering the requirement for beta-2-spectrin for assembly of lateral membranes together with known activities of beta spectrin in binding to phospholipids, the newly described beta-2 spectrin compartment may function in bulk

sorting of lateral membrane lipids.1. Kizhatil, Yoon, and Bennett, ASCB 2005

2674

**The Effects of Chloral Hydrate on Surface Polarity of MDCK Cells**

A. Sandra, C. Overgaard, C. Yeaman; Anatomy & Cell Biology, University of Iowa, Iowa City, IA

The primary cilium, a solitary nonmotile structure projecting from the centriole of all epithelia of the mammalian kidney except the intercalated cells of the collecting duct, serves as a flow sensor in MDCK cells. Removal of the primary cilium by chloral hydrate (CH) treatment abolishes flow sensing in MDCK cells (Praetorius & Spring, J. Membr. Biol. 191:69, 2003). We have treated MDCK cells after reaching confluency (4 days) with 4 mM CH for an additional 3 days. Cells maintained trans-epithelial resistance. In fact, levels averaged approximately 50% higher in the treated cells compared to the non-treated controls. Chloral hydrate treated cells had lost their primary cilia as observed by scanning electron microscopy and fluorescence microscopy in permeabilized cells stained with anti-acetylated tubulin antibody. The microtubule cytoskeleton appeared normal in chloral hydrate treated cells, suggesting that this chemical perturbation can be used to selectively remove the primary cilium. We show that CH treatment of confluent cultures increases cell size, decreases total protein synthesis, but does not significantly affect total protein content. We have also performed cell surface biotinylation experiments to examine steady state localizations of basolateral (E-cadherin) and apical (gp135/podocalyxin) proteins in control and CH-treated cultures. Polarity of gp135/podocalyxin is not significantly affected, but total gp135/podocalyxin accumulation is reduced by > 95%, even though this protein is not present within the cilia. In contrast, neither polarity nor accumulation of E-cadherin is significantly affected by CH treatment. These results suggest that the primary cilium may be specifically linked to gp135 expression, and that its loss leads to changes in cell morphology similar to those promoted by targeted deletion of gp135 reported by others (Meder et al., J. Cell Biol. 168:303, 2005). Supported by NIH DK052617 to C.Y.

2675

**Sec3-Containing Exocyst Complexes Localize to Desmosomes in Polarized Epithelial Cells**

N. Andersen, I. M. Driskell, C. Yeaman; Anatomy & Cell Biology, University of Iowa, Iowa City, IA

The exocyst is a conserved octameric protein complex that functions in targeted delivery of transport vesicles to sites of polarized membrane growth. The last identified subunit of the mammalian complex, Sec3, has been proposed to serve as a spatial landmark for cell polarity in budding yeast. We sought to determine the localization and function of mammalian Sec3 during polarity development in epithelial MDCK cells. In isolated, non-polarized cells polyclonal antibodies label endogenous Sec3 on punctate cytoplasmic structures, but the protein is not associated with plasma membranes. Following induction of E-cadherin-mediated cell-cell adhesion, some Sec3 becomes associated with the basolateral plasma membrane, and colocalizes with desmosomes. This stable localization is distinct from that previously described for Sec6 and Sec8, which are enriched at the zonula adherens (ZA). In order to better understand these localization differences, we compared the immunostaining pattern of 27 different monoclonal Sec6 antibodies. This identified two subsets of antibodies that specifically label Sec6 at either the ZA or desmosomes. The epitopes recognized by these antibodies map to different domains of Sec6. Antibodies to a third exocyst subunit, Sec15, also label desmosomes, suggesting that desmosomes may represent sites of active membrane fusion on lateral plasma membranes. A 200 residue amino terminal domain of Sec3 is ~32% similar to amisyn, a characterized SNARE regulator. In keeping with the localization data, preliminary binding studies show that Sec3NT binds the basolateral t-SNARE syntaxin4 preferentially to the apical t-SNARE syntaxin3. We suggest that distinct Exocyst complexes, characterized by the absence or presence of Sec3, are present at desmosomes and the ZA, and that Sec3 may function to regulate membrane fusion events at desmosomes through interactions with syntaxin4. Supported by NIH GM067002 to C.Y.

2676

**Phosphatidylinositol 3,4,5-trisphosphate Orients the Basolateral Polarity in Epithelial Cells**

A. Gassama,[1] W. Yu,[1] M. Ter Beest,[1,2] K. Mostov[1]; [1]Anatomy, UCSF, San Francisco, CA, [2]The Vontz Center, University of Cincinnati College of Medicine, Cincinnati, OH

Polarity is essential for numerous biological functions including development, migration and differentiation. Epithelial cells are polarized and elaborate a plasma membrane with two distinct domains. The apical domain is specialized for exchange with the lumen and the basolateral membrane maintains contact with adjacent cells and the underlying connective tissue. These two membranes perform different functions and have different protein and lipid composition. How epithelial cells acquire polarity remains a question under intensive study. Phosphatidylinositol (3,4,5)-triphosphate (PIP3) is the major product of Phosphatidylinositol 3-kinase (PI3K) and plays a crucial role in a great number of cellular processes. In polarized MDCK cells, PIP3 is specifically present in the basolateral membrane. Using a lipid shuttle system, PIP3 was added into the apical plasma membrane of polarized MDCK cells. This ectopic localization of PIP3 induces apical membrane protrusions and Akt Phosphorylation. This process required actin polymerisation and activation of Rac 1 and cdc42. Protrusion formation is dependent on the activity of PI3K, suggesting that exogenous PIP3 initiates a positive feed-back loop producing endogenous PIP3 and amplifying the polarization signal. Apical protrusions contained basolateral membrane proteins and excluded apical membrane proteins, indicating that their plasma membrane was transformed from apical to basolateral. Even if we cannot exclude that some of the basolateral proteins found in protrusions are delivered from the Golgi in the biosynthetic pathway, our data indicate that a substantial amount of basolateral plasma membrane components are transcytosed to the apical surface. When MDCK cells are grown in a three- dimensional gel, the cells form hollow cysts lined by a monolayer cells, with their apical surface facing the central lumen. In this system, addition of PIP3 causes formation of basolateral extensions. Our data clearly demonstrate that PIP3 plays a central role in epithelial polarization.

2677

**Requirement for Beta-2-spectrin in Lateral Membrane Formation is Conserved Between Early Mouse Embryo and Cultured Human Bronchial Epithelial Cells**

K. Kizhatil, W. Yoon, V. Bennett; Cell Biology, HHMI and Duke University Medical Center, Durham, NC

Ability of cells to assemble specialized membrane domains is critical for vertebrate physiology. The first formation of polarized membrane domains occurs during compaction of the morula-stage mammalian embryo, and is essential for further development. We previously have

demonstrated that ankyrin-G is required for assembly of lateral membranes in human bronchial epithelial cells (J. Biol. Chem. 179:16706-14, 2004). We now report that ankyrin-G requires interaction with beta-2-spectrin for lateral membrane formation in epithelial cells. Moreover, depletion of beta-2-spectrin by siRNA results in a near complete loss of the lateral membrane without loss of polarity. Exogenous beta-2-spectrin rescues the knockdown phenotype. Beta-2-spectrin is also required for *de novo* lateral membrane biogenesis during cytokinesis. Previous studies have shown that spectrin localizes to sites of cell-cell contact in the early embryo. We show here that beta-2-spectrin is required for formation of lateral membrane in early mouse embryos. Beta-2-spectrin depletion in one cell of a 2-cell embryo by injection of siRNA plasmid into a single blastomere disrupted compaction. Beta-2-spectrin-depleted embryonic cells also fail to complete cytokinesis. Ankyrin-G is co-localized with beta-2-spectrin in embryos. Studies addressing the role of ankyrin-G in embryos are in progress. Together, these results identify beta-2-spectrin as an essential participant in a pathway for assembly of the lateral membrane domain that is conserved between cultured epithelial cells and early embryos.

2678

**Polarized Membrane Trafficking to Pseudopod Tips is Required for Prostate Tumor Cell Motility**

C. E. Overgaard, K. E. Gokay, M. Royer, C. Yeaman; Anatomy & Cell Biology, University of Iowa, Iowa City, IA

Changes in cellular behavior that cause epithelial cells to lose adhesiveness, acquire a motile, invasive phenotype and metastasize to secondary sites are complex and poorly understood. Molecules that function to integrate adhesive spatial information with cytoskeleton dynamics and membrane trafficking likely play important roles in cellular transformation associated with prostate tumorigenesis. The focus of this research project is on specific components of the membrane trafficking machinery (the Exocyst complex and associated SNARE proteins), which are essential for targeted delivery of membrane and secretory proteins to specific plasma membrane sites to maintain epithelial cell polarity. We hypothesize that following loss of E-cadherin expression in prostatic tumor cells, the Exocyst complex responds to distinct spatial cues, assumes a novel localization within protrusive cell extensions, and functions to target the delivery of membrane components required during invasive cell motility. We have determined that several proteins involved in exocytosis (Exocyst, syntaxins3 and 4, Munc18c) are enriched at pseudopod tips of Dunning R3327 prostate carcinoma cells, where they co-localize and co-purify with focal adhesion proteins involved in regulating membrane trafficking and cytoskeleton dynamics (paxillin, b-PIX, GIT1, Nck1/2). These sites are the preferred destination of post-Golgi transport vesicles ferrying biosynthetic cargo, such as a5-integrin and VSVG. We demonstrate that assembly of transport vesicle targeting patches at filopod tips is regulated by two small GTPases (Arf6 and RalA). Interference with expression and activity of these GTPases, or with the Exocyst complex itself, impairs the cargo delivery to the plasma membrane and inhibits cell motility. Overall, we hope that identification of other proteins associated with Exocyst complex at these sites will lead to a deeper understanding of the role that polarized vesicle trafficking plays in cell motility and metastasis. Supported by DOD DAMD17-03-1-0187 to CY.

2679

**Parallel Targeting of the Exocyst Components for Polarized Exocytosis**

X. Zhang, A. Zajac, J. Zhang, W. Guo; Department of Biology, University of Pennsylvania, Philadelphia, PA

The exocyst is an octameric protein complex essential for tethering secretory vesicles at specific domains of the plasma membrane for exocytosis. However, how the exocyst components are targeted to sites of secretion and how they mediate vesicle tethering is unclear. Here we show that distinct mechanisms control the polarization of different components of the exocyst to sites of secretion. The targeting of the exocyst component Sec3p to the bud tip requires its direct interaction with the activated form of Rho GTPases. On the other hand, all the other seven exocyst components (Sec5p, Sec6p, Sec8p, Sec10p, Sec15, Exo70p and Exo84p) are targeted concurrently with the delivery of the secretory vesicles, which depend on polarized actin cables organization. Among these seven components, Exo84p plays a critical role in both the targeting and assembly of this subgroup of exocyst components. Simultaneous disruption of the targeting of Sec3p (at the plasma membrane) and Exo84p (at the vesicle) leads to depolarization of all the exocyst components and results in secretion blocks and cell morphological defects. We further present evidence that vesicle tethering and subsequent SNARE assembly is defective in the double mutants. We propose that this specific targeting mechanism is important for vesicle tethering and polarized exocytosis.

2680

**Tetanus Neurotoxin-mediated Cleavage of Cellubrevin Impairs Epithelial Cell Migration and Integrin-dependent Cell Adhesion**

T. Galli,[1] V. Proux-Gillardeaux,[1] J. Gavard,[2] T. Eirinopoulou,[2] R. Mège[2]; [1]Cell Biology, Institut Jacques Monod, Paris, France, [2]Inserm u706, Institut du Fer-à-Moulin, Paris, France

A role for endocytosis and exocytosis in cell migration has been proposed but not yet demonstrated. Here we show that cellubrevin, an early endosomal v-SNARE, mediates trafficking in the lamellipod of migrating epithelial cells and partially colocalizes with markers of focal contacts. Expression of tetanus neurotoxin, which selectively cleaves cellubrevin, significantly reduced the speed of migrating epithelial cells. Furthermore, expression of tetanus neurotoxin enhanced the adhesion of epithelial cells to collagen, laminin, fibronectin and E-cadherin, altered spreading on collagen, and impaired the recycling of β1 integrins. These results suggest that cellubrevin-dependent membrane trafficking participates in cell motility through the regulation of cell adhesion.

2681

**Axonal Targeting of NgCAM: Testing the Transcytotic Pathway Model**

C. Yap, D. Wisco, J. Cannon, P. Kujala, J. Klumperman, E. Anderson, B. Winckler; Neuroscience, University of Virginia, Charlottesville, VA

Vertebrate neurons are highly polarized cells possessing different processes, namely axons and dendrites. In order to fulfill their distinct functions, axons and dendrites are morphologically and molecularly distinct. Exactly how somatodendritic and axonal membrane proteins accumulate in the correct domains is less than clear, but some sort of targeting mechanisms must exist. We are studying the targeting of the neuronal cell adhesion molecule L1/NgCAM. We proposed previously based on kinetic analysis that NgCAM travels to the axon by an indirect route whereby it is first targeted to the somatodendritic domain, then endocytosed and sorted to the axon from a somatodendritic endosomal compartment. In this work, we first characterize the transport of NgCAM through the endocytic compartments in the somatodendritic domain that accumulate NgCAM, and

secondly, we map the signals required for transcytotic transport. Consistent with NgCAM transcytosis, we provide evidence that 1. NgCAM does not travel to lysosomes after endocytosis, 2. endocytosed NgCAM sorts away from recycling cargo such as transferrin, 3. endocytosed NgCAM in somatodendritic domain highly co-localized with NEEP-21, a neuron-enriched endosomal protein, and 4. the cytoplasmic tail contains multiple signals necessary for transcytotic transport to the axon.

2682

**SARA and Phosphatidylinositiol-3'-phosphate in Disc Morphogenesis and Membrane Trafficking in Mammalian Photoreceptors**

J. Chuang, T. Yeh, C. Sung; Ophthalmogy, Dyson Vision Research Institute, Weill Medical College of Cornell University, New York, NY

The rod photoreceptor is a phototransducing neuron that is highly polarized in both morphology and function. The rod outer segment (ROS), where phototransduction occurs, contains hundreds of membrane discs stacked in an order array. Rhodopsin, the light-absorbing visual pigment of rod cells, is highly concentrated in the ROS. The ROS and its components are continuously and rapidly renewed throughout the lifetime of the animal. The tip of the ROS is regularly phagocytosed by the adjacent retinal pigment epithelium, while at the base of the ROS, new discs are assembled from proteins and lipids synthesized in the cell body (inner segment). Several lines of evidence have clearly demonstrated that the coordination between ROS breakdown and renewal is critical to the health of photoreceptors. We have isolated a FYVE domain-containing protein SARA (Smad Anchor for Receptor Activation) as a rhodopsin C-terminus interacting protein. The FYVE domain of SARA binds specifically to phosphatidylinositiol-3'-phosphate (PI3P), a product of phosphatidylinositiol-3'-kinase (PI3-kinase). Ultrastructural analysis shows that SARA is highly abundant on the vesicles/tubules and sites immediately adjacent to the "nascent" disc membranes in the outer segment portions of axonemes in rodent rod photoreceptors. Suppression of SARA via RNA interference in the rat retina leads to the impairment of ROS targeting of rhodopsin. Overexpression of FYVE domain, which blocks the SARA-PI3P interaction, affects both the ROS distribution of rhodopsin and the disc morphogenesis in transfected rods. Our results, collectively, suggest that SARA and PI3P are critically involved in the membrane targeting of rhodopsin as well as the disc morphogenesis. Finally, our results open discussion for a model explaining the rod disc formation and renewal.

## Developmental Control of Gene Expression (2683-2689)

2683

**Identification of Early TPA-Responsive Genes during Differentiation of HL-60 Cells**

L. Hu,[1,2] C. Tepper,[3] S. Lo,[4] W. Lin[2]; [1]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan Republic of China, [2]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan Republic of China, [3]Department of Biological Chemistry and Cancer Center Basic Science, University of California-Davis,, Sacramento, CA, [4]Department of Orthopedic Surgery and Center for Tissue Regeneration and Repair, University of California-Davis, Sacramento, CA

Human myeloid leukemia cell line, HL-60, has been widely used as a model of inducible macrophage-like differentiation in response to 12-O-tetradecanoylphorbol -13- acetate (TPA). We would like to study early TPA-responsive genes that might act as key regulators of the differentiation of HL-60 cells. We have adopted a special experimental approach in which the cells are synchronized to reduce heterogeneity and are pretreated with a protein synthesis inhibitor, cycloheximide, to distinguish primary and secondary target genes, and microarrays are used to identify genes showing direct regulation of expression by TPA. 62 genes upregulated, and 14 downregulated, by TPA were identified in this study. A subset of the TPA-upregulated genes was further validated by real-time PCR. Three directly TPA-upregulated genes (*NFIL3*, *SKIL*, and *JMJD3*), not previously reported to be TPA-responsive and involved in HL-60 cell differentiation, were shown to be early responsive targets with or cycloheximide pretreatment. TPA upregulation of *SKIL* and *JMJD3* mRNA levels was dose-dependent and also PKC-dependent, as this effect was blocked by the PKC inhibitor, Go6983. Our results show that our method can identify genes as candidate primary targets of the TPA-induced differentiation program.

2684

**Estrogen Activates the Promoter for the PEMT (phosphatidylethanolamine N-methyltransferase) Gene**

J. Song, S. H. Zeisel, S. Wang, K. A. da Costa; UNC, Chapel Hill, NC

Choline is an essential nutrient for humans; however some part of this requirement can be met by endogenous synthesis from 1-carbon groups catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT). Pre-menopausal women are resistant to dietary choline deficiency, while post-menopausal women and men are not. There is evidence in animal models suggesting that estrogen can increase PEMT activity. The aim of this study was to investigate whether and how the PEMT gene is upregulated by estrogen. PEMT gene transcription was increased in a dose-response manner when cultured primary mouse hepatocytes were treated with various concentrations of 17β-estradiol for 24 hours. This preceded an increase in protein expression and enzyme activity. Transfection studies, in which the human PEMT promoter was linked to a luciferase reporter, demonstrated that estrogen significantly increased promoter activity. This effect was reversed by ICI 182,780, an estrogen antagonist. The human PEMT promoter contains eight putative estrogen response elements (EREs), and Electrophoretic Mobility Shift Assay revealed that a 41 bp segment which contains two putative EREs can bind estrogen receptor complexes. We have previously reported that there is a common single nucleotide polymorphism (SNP) in the PEMT promoter, and that this SNP (rs12325817) increases susceptibility of women to developing signs of choline deficiency. Using EMSA we showed that the SNP is in a region that is estrogen responsive. And the SNP abrogates this responsiveness. This study is the first to explore the underlying mechanism of why premenopausal women are relatively resistant to choline deficiency.

2685

**Human *NR4A2* (Nurr1) *cis*-Regulatory Sequences Identified by Comparative Sequence Analysis and a Functional Screen in Zebrafish**

R. C. Addis,[1,2] R. M. Vinton,[2] S. Fisher,[2] A. S. McCallion,[2] J. D. Gearhart[1]; [1]Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, [2]McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD

Directed differentiation of stem cells requires an understanding of the mechanisms that control critical gene expression during development. Several genes have been identified as key players in the development of midbrain dopaminergic neurons, the neurons that are destroyed in Parkinson's Disease. *NR4A2* (Nurr1) is essential for the genesis and survival of midbrain dopaminergic neurons, but little is known about the

mechansims by which the transcription of this gene is regulated. Recent reports have demonstrated that evolutionarily conserved non-coding sequences within and flanking a gene are likely to contain *cis*-acting regulatory sequences. We have compared the sequence at the human *NR4A2* locus with its orthologs from multiple vertebrate species to identify non-coding regulatory sequences at this gene. We tested putative regulatory sequences for *in vivo* function by placing them upstream of the *c-fos* minimal promoter driving GFP and inserting this construct into the zebrafish genome using the Tol2 transposase system. Transgenic zebrafish were examined for GFP expression in regions of the central nervous system known to express the zebrafish ortholog of *NR4A2*, as determined by *in situ* hybridization. We report the identification of a conserved non-coding sequence upstream of *NR4A2* exon 1 that is sufficient to drive expression in a pattern that recapitulates much of the zebrafish Nurr1 expression pattern. We have begun experiments using this sequence to drive reporter gene expression in *NR4A2*-expressing dopaminergic neuron precursors derived from embryonic stem cells to facilitate their isolation using flow cytometry. In addition, we aim to identify the transcription factors that bind to this *cis*-acting sequence. Our goal is to efficiently direct the differentiation of embryonic stem cells into dopaminergic neurons via ectopic expression of identified critical transcription factors.

2686

**Nitric Oxide Activates Peroxisome Proliferator-Activated Receptor Gamma through a p38 MAPK Signaling Pathway**

A. Ptasinska, S. Wang, J. Zhang, R. L. Danner; National Institutes of Health, Critical Care Medicine Department, Clinical Center, Bethesda, MD

Nitric oxide (NO) regulates vascular tone, gene expression, platelet aggregation and leukocyte adhesion. Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors that have functions which overlap with those of NO such as the repression of endothelin-1 and vascular adhesion molecules. All three PPARs PPARa, PPARb/d and PPARg are expressed in endothelium. Since both NO and PPARg regulate endothelial function and protect the endothelium from injury, we hypothesized that these signaling pathways may interact with each other in the vasculature. Human-hybrid endothelial cells (EA.hy 926) were exposed to NO donors S-nitrosoglutathione (GSNO) or spermine NONOate. Electrophoretic mobility shift assays using PPAR-response element (PPRE) probe showed that low dose NO exposure (100 microM) increases PPARg binding. The activation of PPARg binding reached a maximum after 30 to 60 minutes of exposure to NO and was sustained for at least 4 hours. NO had no effect on PPARg protein expression level as detected by Western blotting. GSNO or NONOate was found to activate p38 MAPK in EA.hy 926 cells and p38 MAPK inhibitor, SB202190 (0.1 microM), or siRNA knockdown of p38 MAPK were both shown to block the effect of NO on PPARg binding. Using a luciferase-based reporter gene system, NO activated PPRE containing promoter of cyclooxygenase-2 (COX-2) and a PPRE artificial construct. Further, NO was found to induce the expression of PPRE containing genes including COX-2 and diacylglycerol kinase a (DGKa). These results show that NO increases PPARg binding and activates PPRE containing promoters through a p38 MAPK signal transduction pathway. Thus, NO may exert anti-inflammatory and cytoprotective effects in the vasculature through PPARg. This interaction can be used to identify new of PPARg target genes and potentially may lead to new endothelium protective strategies.

2687

**Developmental Expression Profile and Transcriptional Regulation Analysis of Murine Pinin**

S. Leu, P. Ouyang; Department of Anatomy, Chang Gung University, Tao-Yuan, Kwei-Shan, Taiwan Republic of China

Pnn is a multifunctional protein with dual subcellular localization at desmosomes and in nuclear speckles. The involvement of pnn in mouse development and the mechanism underlying the regulation of pnn expression are still unknown. To characterize the role of pnn in mouse embryo development, we monitored distribution patterns and expression levels of pnn in mouse embryos of each developmental stage. With Northern blot and Real-Time RT-PCR, we found that E12.5 embryos displayed the highest expression of pnn and the expression levels then declined gradually till birth. Immunostaining and mRNA in situ hybridization showed that pnn is widely expressed and its expression is detected in as early as 2-cells stage mouse embryo. Tissues with higher expressions of pnn during mouse development include CNS, E12.5 to E14.5 livers, post-E13.5 epidermis, and E16.5 thymus. To investigate the regulatory mechanism for pnn expression, we determined the promoter region of mouse pnn gene and found regions critical for pnn expression regulation. Through luciferase activity assay on truncated pnn promoter fragments, we determined a region 200 to 180 bp upstream of ATG start codon containing an unidentified *cis*-acting element which plays an important role in enhancing the promoter activity of mouse pnn. In addition, we found that the Ets consensus binding site GGAA at 151 to 148 bp upstream of translation start site is essential for basal pnn expression and is regulated by the transcription factor GABP.

2688

**Design of siRNAs that Silence the Pain Gene PKCγ**

M. Lozada Contreras,[1] S. Garraway,[2] C. E. Inturrisi[2]; [1]Industrial Biotechnology, University of Puerto Rico, Mayaguez Campus, Mayaguez, Puerto Rico, [2]Pharmacology, Weill Medical College of Cornell University, NY, NY

RNA interference is a powerful genetic approach for efficiently silencing target genes. One advantage of this technique is the high specificity it offers over pharmacological antagonism in that it cleaves the mRNA of a precise protein. The purpose of this study is to use RNA interference to target PKCγ, a protein kinase that has been identified in neurons of the brain and spinal cord and implicated in the production of persistent pain. Firstly, we designed five 21 base pair small interfering RNAs (siRNA) that target the gene that encodes the mouse PKCγ by using the MIT-Whitehead siRNA design program. Next, the siRNAs were subjected to a NCBI BLAST search which checks the specificity of each siRNA for the targeted gene and identifies similar sequences in other genes. To screen for effective siRNAs, a small hairpin RNA (shRNA) will be constructed from potential siRNAs by the addition of a stem loop structure. The shRNA is then cloned into the pAAV plasmid with a H1 promoter. Subsequently the PKCγ cDNA (mouse) is inserted into the psiCHECK vector, containing the reporter genes renilla luciferase (Rluc) and firefly luciferase. Both plasmids will be co-transfected into HEK 293 cells. Effective shRNAs will cleave the PKCγ cDNA, which will decrease the luminescence of Rluc in comparison to the firefly. Five siRNAs were tested; only three yielded colonies on plates containing selective growth media. Following several restriction digestion analysis, two siRNAs are confirmed to be transfected into HEK 293 cells. Effective shRNA will be incorporated into viral vectors to be injected into animals. A successful PKCγ siRNA will prevent or reduce pain in a mouse model.

2689

**Regulated Cell Differentiation: Expression of the Traits "Mating type" and "Phagotype" in Ciliate *Dileptus anser***

M. Tavrovskaja, S. Vassyanin; Lab.Cytology of the Unicellular Organisms, Institute of Cytology Academy of Scienses of Russia, Saint Petersburg, Russian Federation

Ciliates exhibit cell differentiation by "mating type"(MT)(Sonneborn, 1937). Trait MT is expressed at particular stage of clone life cycle. Cells of *Dileptus anser* preserves some features of parent MT during the first 24 h after completion of conjugation process. Immaturity period (IP) lasts several months. Maturity begins with secretion of gamone. Experiments show dependence of the duration of immaturity period from number cell division. Two types of fading regime has been offered to the cells of the same exconjugant clone-the first provided intensive cell fission (regime I), cells under the second regime practically did not divided (regime II). Maturity testing of the cells in two groups cultivated during the same physically time has demonstrated maturity of divided cells (regime I) and immaturity of undivided cells (regime II). The very high regenerative ability and peculiarity of nuclei structure *D. anser* cells gave us opportunity to shorten IP to a greater extent by growing cells from caudal mini-fragments of operated cells (Tavrovskaya,1981). Clones originated from mini-fragments of the cell had the same MT as not operated cells after maturing.. No shortening of IP has been found in the cells which originated from macro-fragments of operated cells. Another induction-receptor system of Dileptus anser's cells regulates self- identification and intraclonal fade behavior and determinate the trait phagotype (PhT), (Tavrovskaya,1977). Exconjugants preserved parents PhT nearly 24 h after conjugants separation; in 30h of exconjugants life new PhT was expressed. PhT changing occured without exogenic nutrition, prior to somatic nuclei formation and first cell fission. Testing exconjugants and parents clones during 3,5 years has shown stability of the trait PhT and independence PhT and MT systems. .

## Ribonucleoproteins (2690-2703)

2690

**Gemin Proteins Are Required for Efficient Assembly of Sm-class RNPs**

K. B. Shpargel, A. G. Matera; Genetics, Case Western Reserve University, Cleveland, OH

Small nuclear ribonucleoprotein (snRNP) biogenesis constitutes a pathway wherein the uridine-rich small nuclear RNAs (UsnRNAs) that mediate pre-mRNA splicing are complexed with proteins, modified and transported to their appropriate subnuclear locales. The survival of motor neurons (SMN) protein, along with Gemins2-7, forms a large oligomeric complex that is necessary and sufficient for the in vivo assembly of Sm proteins onto snRNAs. Following cytoplasmic assembly of the Sm core, both SMN and splicing snRNPs are imported into the nucleus, targeting Cajal bodies for additional snRNA maturation steps before accumulating in splicing factor compartments known as "speckles." In this study, we have analyzed the function of individual SMN complex members by RNA interference (RNAi). Notably, we find that RNAi-mediated knockdown of SMN, Gemin2, Gemin3 and Gemin4 each disrupt Sm core assembly, whereas knockdown of Gemin5 and Snurportin1 had no effect. Assembly activity can be rescued by expression of a GFP-SMN construct that is refractive to RNAi, but not by similar constructs that contain mutations that cause Spinal Muscular Atrophy in humans. Our results also demonstrate that Cajal body homeostasis requires ongoing snRNP assembly by the SMN complex. Perturbation of SMN complex function results in disassembly of Cajal bodies and relocalization of the marker protein, coilin, to nucleoli. Moreover, newly-synthesized SmB proteins fail to accumulate in Cajal bodies or speckles in SMN-deficient cells. Importantly, the work reveals a novel function for Gemin3 and Gemin4 in Sm core assembly and suggests that defects in snRNP biogenesis contribute to SMA pathogenesis.

2691

**Arginine Methylation and Small Nuclear Ribonucleoprotein Biogenesis : Re-examining the Connection**

G. B. Gonsalvez, T. K. Rajendra, A. G. Matera; Genetics, Case Western Reserve University, Cleveland, OH

Post-translational modification of proteins is a common mechanism used by cells to regulate function. One type of post-translational modification, protein methylation, regulates a vast array of cellular functions. Studies have demonstrated that proteins involved in carrying out pre-messenger RNA splicing are dimethylated on specific arginine residues. These splicing factors are called small nuclear ribonucleoproteins (snRNPs) and the enzymes that modify and potentially regulate the assembly of snRNPs are called protein arginine methyltransferases (PRMTs). Under *in vitro* conditions, the snRNP proteins SmB/B', SmD1 and SmD3 can be symmetrically dimethylated by either PRMT5 or PRMT7. It is unknown which of these enzymes, PRMT5 or PRMT7, is responsible for methylating Sm proteins *in vivo*. Furthermore it is unknown whether dimethylation of Sm proteins is required for proper snRNP maturation and splicing. To gain an *in vivo* understanding of this process, we carried out our studies using *Drosophila melanogaster*. We characterized an allele that contained a disruption in the *Dart5* gene, the fruit fly ortholog of human PRMT5. We demonstrate that in the absence of Dart5, Sm proteins are completely un-methylated, indicating that Dart5 is required for Sm protein methylation *in vivo*. Surprisingly, the absence of Sm methylation does not affect the viability of F1 homozygotes. However, *Dart5* is an essential gene as the homozygous mutant animals are sterile. Interestingly, the absence of Sm protein methylation does not adversely affect snRNP biogenesis. These findings suggest that methylation of Sm proteins is required for a non-splicing related function. Consistent with this interpretation, Sm proteins, independent of their role in splicing, are required for the proper localization and integrity of germ granules in *C. elegans*.

2692

**RNAi of *Drosophila* Nopp140 Expression is Analogous to the Human Treacher Collins Syndrome**

Z. Cui, P. DiMario; Biological Sciences, Louisiana State University, Baton Rouge, LA

Nopp140 is a nucleolar protein that functions in pre-rRNA transcription or ribosome assembly. Although its functions have not been precisely determined, Nopp140 interacts with both box C/D and box H/ACA snoRNP complexes, thus suggesting a chaperone function in pre-rRNA processing and ribosome assembly [Yang *et al*. (2000) *Mol. Biol. Cell* **11**:567-577]. By alternative splicing, *Drosophila* expresses potentially two isoforms of Nopp140: the first is homologous to mammalian Nopp140, sharing a conserved carboxy terminus. The second contains a distinctive glycine and arginine rich (RGG) carboxy terminus typically found in vertebrate nucleolin. To further characterize Nopp140 in *Drosophila*, we expressed interfering RNAs in transgenic flies from yeast GAL4 upstream activating sequences when crossed to GAL4 driver lines. Western blot analysis showed a dramatic loss of Nopp140 in larvae induced to express RNAi. Larval imaginal discs and histoblasts give rise to adult structures

(wings, legs, and cuticle) during pupation. These larval tissues are the likely targets for Nopp140 loss; adult phenotypes resulting from Nopp140 loss include shriveled wings, deformed legs, and fused tergites (abdominal stripes of the dorsal cuticle). Phenotypes attributed to the loss of Nopp140 are complex and variable, and they fall within the *Minute* syndrome of *Drosophila* that presumably results from a lack of ribosome production and protein synthesis. The *Minute* syndrome may be analogous to the human Treacher Collins syndrome (TCS). TCS displays craniofacial birth defects that are also complex and variable. TCS results from mutations in the *TCOF1* gene that encodes Treacle, a nucleolar protein very similar to Nopp140 in peptide domain organization [e.g. Valdez *et al.* (2004) *Proc. Natl. Acad. Sci.* **101**:10709-10714]. *Drosophila* should be an ideal model system to examine nucleolar protein function during critical periods in development.

2693

**RNA-dependent Nucleolar Sequestration and Suppression of APOBEC3C Deaminase for Avoiding Random Cytosine-deamination of Genomic DNA**

W. HE,[1] R. Chen,[2] Z. Yang,[1] Q. Zhou[1]; [1]Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [2]Xiamen University, Xiamen, China

APOBEC3C is the most widely expressed member of the APOBEC family of proteins that function in diverse biological processes through deaminating deoxycytosine on single-stranded DNA. It is not clear how its activity is regulated so as to prevent the introduction of uncontrolled hypermutations into genomic DNA. Through affinity-purification of proteins that interact with the evolutionally conserved 7SK snRNA, we have identified APOBEC3C as a major 7SK-associated protein. Most of cellular APOBEC3C interact with 7SK in a single complex that also contains the 5S and 5.8S rRNA. Interactions with these RNA species suppress the deaminase activity of APOBEC3C and sequester the protein in the nucleolus. Because the DNA substrate-binding site in APOBEC3C is different from the region required for the APOBEC3C-RNA interaction, we propose that the RNA molecules serve as negative regulators rathar than substrate-competitors in causing the inhibition of APOBEC3C. We discuss the implications of the RNA-dependent regulation of APOBEC3C for the cellular mechanism of preventing deleterious hypermutations caused by this deaminase.

2694

**Initial Characterization of Raver2, a New Member of the HnRNP Family**

B. Kleinhenz,[1] M. Fabienke,[2] H. H. Arnold,[2] B. M. Jockusch,[1] S. Illenberger[1]; [1]Zoological Institute, Cell Biology, Technical University of Braunschweig, Braunschweig, Germany, [2]Cell and Molecular Biology, Institute for Biochemistry and Biotechnology, Technical University of Braunschweig, Braunschweig, Germany

In database searches we identified a novel member of the hnRNP family reminiscent of the previously described raver1 protein. The open reading frame encodes a protein of 625 amino acids. The deduced amino acid sequence revealed highest sequence homology within three RNA recognition motifs and a similar overall organization of functional domains as compared to raver1, prompting us to name the protein raver2. The protein harbours three N-terminal RNA recognition motifs (RRM), two putative nuclear localization signals (NLS) at the N- and C-termini and a central leucine-rich region which may function as a nuclear export sequence. Like raver1, raver 2 displays nucleo-cytoplasmic shuttling in an interspecies heterokaryon assay. However, while raver1 is expressed ubiquitously both in embryonic and adult mouse tissues, raver2 exerts a distinct spatio-temporal expression pattern. During embryogenesis (E8.5) raver2 expression begins in neuroepithelium and continues in dorsal regions of fore-, mid-, and hindbrain. The protein is also expressed in dorsal root ganglia, branchial arches, and the developing limb buds. In adult mice, raver2 is essentially restricted to brain, lung, and kidney. As revealed by immunofluorescence analyses of glia cells and primary neurons, raver2 resides in the nucleus of both cell types, where it co-localizes with the polypyrimidine tract binding protein (PTB) in the perinucleolar compartment, an RNA-dependent structure characterized by the accumulation of PTB. Immunoprecipitation experiments and biochemical analyses demonstrate that raver2 directly binds to PTB. At present, we are investigating, whether raver2 affects PTB-mediated splicing, as has already been implicated for raver1. The different expression patterns, however, imply that both raver proteins have distinct or only partially overlapping cellular functions, possibly during differentiation processes.

2695

**hnRNP A2 Expression is Required for HIV-1 Genomic RNA Trafficking from the Microtubule Organizing Center**

K. Lavesque,[1] M. Halvorsen,[1] L. Chatel-Chaix,[1,2] L. Abrahamyan,[1] V. Poupon,[1] A. Gatignol,[1] A. J. Mouland[1]; [1]Lady Davis Institute for Medical Research/McGill University, Montreal, PQ, Canada, [2]Biochemistry, Université de Montréal, Montreal, PQ, Canada

Few details are known about how the human immunodeficiency virus type 1 (HIV-1) genomic RNA is trafficked in the cytoplasm. We identified heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) as one of the first host proteins involved in this process such that its interaction with cis-acting sequences of the HIV-1 RNA is important for HIV-1 RNA localization, protein expression patterns and levels of genomic RNA in new virions. In order to further define the role of hnRNP A2 in the fate of HIV-1 genomic RNA in the cytoplasm, we used small interfering (si)RNA to knockdown hnRNP A2 gene expression in HIV-1-expressing HeLa cells. In this study we demonstrate that HIV-1 RNA trafficking from the microtubule organizing center (MTOC) is dependent on the expression levels of hnRNP A2. A 90-95% knockdown of hnRNP A2 expression resulted in the perinuclear accumulation of HIV-1 genomic RNA at the MTOC as shown by fluorescence in situ hybridization and immunofluorescence co-analyses. Kinetic studies that allow us to follow the fate of HIV-1 RNA reveal that the accumulation of genomic RNA at the MTOC occurs very rapidly and that HIV-1 genomic RNA egress within the cytoplasm is dependent on hnRNP A2 expression. Western and RT-PCR results show that HIV-1 Gag expression is not affected by siRNA treatment and the reduction of hnRNP A2 expression does not result in aberrant splicing of HIV-1 RNA. The results from this study demonstrate that the cytoplasmic trafficking of the HIV-1 genomic RNA is mediated directly by the activity of the host cell protein, hnRNP A2. These data also indicate that the MTOC is likely the point of convergence for HIV-1 genomic RNA following its exit from the nucleus.

2696

**Polypyrimidine Tract Binding Protein Blocks the 5' Splice Site Dependent Assembly of U2AF and the Prespliceosomal E Complex**

S. Sharma,[1] A. Falick,[2] D. Black[3]; [1]HHMI, UCLA, Los Angeles, CA, [2]UC, Berkeley, San Francisco, CA, [3]HHMI/MIMG, UCLA, Los Angeles, CA

Polypyrimidine tract binding protein (PTB) controls the splicing of many alternatively spliced exons, including the c-src exon N1. The repression of N1 splicing in non-neural cells requires the binding of PTB to multiple sites surrounding the N1 exon. We show that PTB blocks splicing prior to formation of the pre-spliceosomal Early (E) complex. To understand the nature of the pre-mRNP complexes that direct changes in spliceosome assembly, we purified and characterized the regulatory pre-spliceosomal H complexes that assemble onto N1 exon RNAs. The repressed complex contains PTB and lacks the essential splicing factor U2AF, even though the 3' splice site for the unregulated exon is present and far downstream of the PTB binding sites. In contrast, an H complex that can go on to splice contains U2AF. Immunodepletion of PTB from extracts or mutation of a PTB binding site allows binding of U2AF and splicing derepression. It has been shown by others that assembly of U2AF into the E complex requires an interaction with the U1 snRNP. We find that U2AF binding to the downstream 3' splice site is restored by including an unregulated 5' splice site upstream from the N1 exon. Our results indicate that PTB represses splicing by blocking an interaction of U2AF with the 5' splice site, presumably via the U1 snRNP that is required for U2AF assembly. Thus, rather than simply occluding a binding site for U2AF, this splicing repressor blocks a very specific interaction required for U2AF assembly into the early spliceosome.

2697

**The Paraneoplastic Neurologic Disease Antigen Nova is a Shuttling Protein and May Have Dual Functions in Nucleus and Cytoplasm**

T. Eom,[1] M. Ruggio,[1] R. Darnell[1,2]; [1]Lab of Molecular Neuro-oncology, The Rockefeller University, New York, NY, [2]HHMI, New York, NY

RNA binding proteins are important for the regulation of transcription, alternative splicing, translation and localization of mRNAs. Nova proteins are neuronal KH-type RNA binding proteins and are targeted in paraneoplastic opsoclonus myoclonus ataxia (POMA). It has been known that Nova proteins play a role as a splicing factor in neurons. Immunohistochemical studies have demonstrated that Nova proteins are highly expressed in the nucleus, as well the cytoplasm. Additionally, sucrose gradient fractions revealed that the Nova proteins comigrate with polysomes in mouse brain. These data strongly suggest that Nova proteins may have some functions in the cytoplasm besides being a splicing factor in the nucleus. Moreover, Nova proteins have both a Nuclear Localization Signal (NLS) as well as Nuclear Export Signal (NES). They may direct the bi-directional transport of Nova proteins between the nucleus and cytoplasm and provide different functions in different areas. A new method, CLIP (in vivo cross-linking IP analysis) has identified many in vivo RNA targets of Nova proteins through covalent cross-linking Nova proteins and RNA complexes without any in vitro manipulation. Immunohistochemical and fluorescence in situ hybridization (FISH) studies may define specific expression patterns of these Novas' target genes such as Gephyrin, Girk2 and CamKII g in discrete regions of the developing nervous system by comparing the difference of distribution, stability and protein expression levels of RNA targets in wild type and Nova-null mice. These studies will demonstrate the physical interaction between Nova proteins and RNA targets and the significance of this interaction in neurobiology.

2698

**Homotrimerization of the Trim Protein Pwa33/xnf7 is Required for its Association with Active Transcription Units**

B. Beenders, M. Bellini; Cell & Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL

In amphibian oocytes, the TRIM (tripartite motif) protein PwA33/Xnf7 associates with most loops of the lampbrush chromosomes. These loops correspond to active transcription sites of RNA polymerase II, which are visible by light microscopy because of their thick ribonucleoprotein (RNP) matrix. While PwA33/Xnf7 was described as a component of the loop matrix, about half of the nuclear supply of the protein remains free in the nucleoplasm. Fractionation of an oocyte extract by chromatography indicated that the predominant form of PwA33/Xnf7 is a homotrimer. This data was further supported by glutaraldehyde cross-linking of the endogenous and *in vitro* made PwA33/Xnf7. In addition, deletion analyses demonstrated that the putative coiled-coil region of the protein is essential for its trimerization. Finally, we demonstrated that a coiled-coil-deleted PwA33/Xnf7 failed to associate with chromosomes. Together, these data strongly suggest that PwA33/Xnf7 interact with active transcription units as a homotrimer and provide new insights into the structural role of its TRIM motif.

2699

**Uncovering the Mechanism of Effective Diced Pools for RNAi**

D. Gong,[1] J. W. Myers,[2] J. E. Ferrell[3]; [1]Biological Sciences and Molecular Pharmacology, Stanford University, Stanford, CA, [2]Biochemistry, Stanford University, Stanford, CA, [3]Molecular Pharmacology, Stanford University, Stanford, CA

The use of RNA interference (RNAi) for loss-of-function studies has recently gained popularity due to the rapid optimization of techniques for triggering gene silencing in mammalian cells. Generating synthetic siRNAs, and DNA-based vectors as RNAi triggers generally require screening two to four siRNAs before identifying one that is effective for silencing while the rest seem to have little to no effect. Use of Dicer-generated siRNAs (d-siRNAs) bypasses this screening requirement, and is consistently effective for gene silencing (>80% silencing, 80% of the time). Since d-siRNAs consist of presumably large numbers of siRNAs, we explored two possibilities by which diced pools achieve consistent and effective silencing: (i) siRNAs within the pool work in a cooperative fashion regardless of their individual efficacies, or (ii) highly effective siRNAs exist that confer the silencing ability of the pool, despite other ineffective siRNAs around. To address these possibilities, we identified the complexity of a diced pool by cloning, sequencing, then selected a subpopulation of these siRNAs to make small pools to try to reconstitute the efficiency of the original diced pool. We found that a diced pool generated against a 1.3 kb region of our target gene consists of at least 336 unique siRNAs. When we tested 10 of these single siRNAs for silencing, or when different combinations of siRNAs were mixed together as small pools, no single or combination silenced better than the original pool. Our data indicates that individual siRNAs do not exhibit cooperativity. Although we have not identified an siRNA that is better than original pool for silencing, it seems that nature has chosen dicing as a method of generating a large number of siRNAs to ensure that at least some siRNAs will be potent for silencing.

2700

**The Structurally Diverse Protein L23a of *Drosophila melanogaster* is a Functional Member of the L25/L23 Ribosomal Protein Family**

C. L. N. Ross, R. Patel, V. C. Ware; Biological Sciences, Lehigh University, Bethlehem, PA

The ultimate products of the ribosome biogenesis pathway are ribonucleoprotein complexes that function in protein synthesis. Some ribosomal components are remarkably well conserved in structure between species, highlighting their functional significance within the biogenesis pathway. Many ribosomal components are less well conserved or are unique to certain lineages. The utilization of conserved and unique structural

components to construct the protein synthetic machinery is a notable evolutionary feat. The proposed *Drosophila melanogaster* L23a homologue is a structurally unique ribosomal protein compared to the widely studied L25 protein from *Saccharomyces cerevisiae*. The fly L23a protein includes an N terminal extension, creating a protein of 277 amino acids in length compared to 142 amino acids for yeast L25. The ability of fly L23a with an N-terminal extension to replace yeast L25 function was determined by creating a *S. cerevisiae* strain carrying an endogenous L25 chromosomal gene disruption and a plasmid-encoded FLAG-tagged L23a gene. The yeast strain is dependent on the function of a FLAG-tagged fly L23a for survival and growth, demonstrating functional compatibility between fly L23a and yeast L25. Fly L23a-FLAG is incorporated into yeast ribosomes, as demonstrated by affinity purification of ribosomes using an anti-FLAG antibody. Interestingly, in yeast strains that carry both yeast and fly L25/L23a genes, fly L23a RNA is produced but fly L23a protein does not accumulate, suggesting that fly L23a protein is rapidly degraded within these strains and does not compete effectively with endogenous L25 for assembly into yeast ribosomes. *Supported by PA Department of Health Block Grant and Lehigh University.*

2701

### *Lacandonia* Granules during Mitosis

L. F. Jiménez-García,[1] M. L. Segura-Valdez,[1] J. Otero,[1] F. Fernández,[1] A. M. Castrejón,[1] J. Jiménez-Ramírez[2]; [1]Cell Biology, UNAM, Mexico City, Mexico, [2]Comparative Biology, UNAM, Mexico City, Mexico

*Lacandonia* granules are intranuclear ribonucleoprotein particles present and first observed in the nuclei of the plant *Lacandonia schismatica.* They are also observed in other species as *Triuris brevistylis* and *Ginkgo biloba.* These particles contain poly-A RNA and SR proteins and their number changes during development. Here we present additional biological aspects of *Lacandonia* granules. Ultrastructural cytochemical procedures reveal that they are present in all tissues of *L. schismatica* as well as in several other plant species. In addition, analysis of these granules throughout mitosis in three different species indicates that their number decreases as mitosis progresses. At telophase, their number increases again. Taking together, our results show that *Lacandonia* granules are conspicuous ribonucleoproteins in plants and the behavior during mitosis suggests they are not inherited from one cell cycle to the next. Supported by DGAPA-UNAM IN-221202

2702

### Isoproterenol Attenuates Wound Healing in Airway Epithelium via cAMP Signaling

B. J. Schnackenberg,[1,2] S. M. Jones,[1,2] C. Pate,[1] B. Shank,[1] L. Sessions,[1] L. M. Pittman,[1] L. E. Cornett,[3] R. C. Kurten[3,2]; [1]Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, [2]Arkansas Children's Hospital Research Institute, Little Rock, AR, [3]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR

β-agonists constitute a major group of medications used to treat the bronchospasms associated with asthma. It has been shown that β-agonists can trans-activate epidermal growth factor (EGF) receptors in a number of cell types. Since EGFRs are involved in wound healing, we hypothesized that β-agonists would have an effect on wound healing. Therefore, the objective of this study was to determine the effects of β-agonists on airway wound healing and the signaling pathways involved. We used human bronchial epithelial (16HBE) cell cultures as a model for airway wound healing. Serum-starved cultures were scrape wounded and cultured in media containing 1% fetal bovine serum (FBS) supplemented with various drugs. Percent wound closure was determined by measuring the wound areas in images collected after 6 hours. Wounds treated with EGF healed quicker than those treated with FBS alone (90% vs. 67% closure, P=0.014) and this stimulation was attenuated by isoproterenol (ISO) (72%, P=0.041). Using Western blot analysis, we show that the attenuation does not occur by directly affecting EGFR activity or ERK signaling. To determine the effects of cAMP on this attenuation, we used the adenylyl cyclase activator forskolin (FOR) to increase cAMP levels. Wounds treated with FBS+EGF+FOR closed slower than those treated with FBS+EGF (61% vs. 93%, P<0.001) or FBS+EGF+ISO (84%, P<0.001). Finally, over-expression of $\beta_2$-adrenergic receptors ($\beta_2$ARs) has been shown to increase cAMP production upon receptor activation. 16HBE cells stably expressing $\beta_2$AR-YFP closed wounds significantly slower when treated with FBS+ISO vs. FBS alone (42% vs. 68%, P<0.001) or when treated with FBS+EGF+ISO vs. FBS+EGF (52% vs. 85%, P=0.013). Based on this data, we conclude that ISO attenuates the EGF-stimulated wound healing in airway epithelium through cAMP-dependent signaling pathways activated by $\beta_2$ARs.

2703

### A Repeated IMP-binding Motif Controls *oskar* mRNA Translation and Anchoring Independently of *Drosophila* IMP

S. Kwon,[1] T. P. Munro,[2] D. St Johnston,[2] B. J. Schnapp[1]; [1]Cell & Developmental Biology, Oregon Health & Science University, Portland, OR, [2]The Gurdon Institute, University of Cambridge, Cambridge, United Kingdom

Zipcode binding protein-1 (ZBP-1) and its *Xenopus* homologue, VERA/Vg1RBP, selectively bind repeated elements in the 3'UTRs of some asymmetrically localized mRNAs. Although the repeated motifs are required for localization of these RNAs, the requirement of ZBP-1/VERA remains unresolved. Here we address the role of ZBP-1/VERA in mRNA localization through analysis of the *Drosophila* homologue, IMP. Using SELEX with the IMP KH domains, we identified the IMP binding element (IBE), UUUAY, and found that this motif occurs 13 times in the *oskar* 3'UTR. IMP co-localizes with *oskar* mRNA throughout oogenesis, and this depends on the IBEs in the RNA, since IMP is de-localized by single nucleotide substitutions in all of the *oskar* IBE motifs. These point mutations do not affect the initial localization of *oskar* mRNA, but prevent its translational activation and anchoring at the posterior pole. Furthermore, mutations to non-overlapping subsets of 3 or 4 of the 13 IBEs also block translation and anchoring completely. However, *oskar* mRNA is localized and translated normally in germline clones of an imp null mutation. Thus, *oskar* regulation requires the IMP binding elements, but not IMP itself. Although many factors have been shown to play a role in the translational repression of unlocalized *oskar* mRNA, much less is known about how translation is activated once the mRNA is localized at the posterior pole. Here we show that this translational de-repression requires multiple copies of the IBE, establishing a parallel requirement for this repeated motif in the regulation of localized maternal mRNAs in *Drosophila* and *Xenopus*. Since IMP itself is dispensable in the germline, translational activation of *oskar* mRNA must depend on another factor that recognizes IBEs. (S.K. and T.P.M. contributed equally, and D.S.J. and B.J.S. contributed equally.)

## Structure of Nuclear Envelope (2704-2721)

2704

### Nesprin-2: An Essential Giant Scaffolder of the Nuclear Envelope

H. Zaim,[1] Y. Lüke,[1] M. Munck,[1] M. Wehnert,[2] G. Walz,[3] A. Noegel,[1] I. Karakesisoglou[1]; [1]Institute for Biochemistry I, Medical Faculty, University of Cologne, Cologne, Germany, [2]Institute of Human Genetics, Ernst-Moritz-Arndt-University, Greifswald, Germany, [3]University Hospital Freiburg, Department of Medicine, Nephrology, Freiburg, Germany

The vertebrate proteins Nesprin-1 and Nesprin-2 together with ANC-1 of *C. elegans* and MSP-300 of *D. melanogaster* belong to a novel family of α-actinin type actin binding proteins residing at the nuclear membrane. Recently, we established linkages and interconnections between Nesprins, lamin A/C and emerin. While the Nesprin-2 subcellular distribution depends upon the lamin A/C network, emerin itself requires Nesprin-2 for proper localization at the nuclear envelope. Lamin A/C mutations may therefore disrupt the localization and function of Nesprin-2 at the nuclear envelope. In agreement with such a scenario we report that the S143F lamin A/C mutation, which causes an early-onset myopathy and progeria in humans, affects specifically the localization of Nesprin-2. Interestingly the expression levels and the subcellular localization of Nesprin-1 is unaltered in the progeroid cells, suggesting that while Nesprin-1 and -2 are similar in their domain architecture they may be functionally divergent. We observed a miss-localization of Nesprin-2 in 40% of the mutant cells, which was accompanied by dramatic nuclear defects. Similar data where obtained in Nesprin-2 knockout mouse dermal fibroblasts. Loss of Nesprin-2 resulted in nuclear deformations and in the miss-localization of emerin. Our data imply a scaffolding function of Nesprin-2 at the nuclear membrane and suggest a potential involvement of this multi-isomeric protein in human disease.

2705

### Structural Analysis of the Domain Topology of the Nucleoporin Nup214/CAN Depending on the Conformational State of the Nuclear Pore Complex

S. M. Paulillo,[1] M. A. Powers,[2] K. S. Ullman,[3] B. Fahrenkrog[1]; [1]M.E. Müller Institute for Structural Biology, University of Basel, Basel, Switzerland, [2]Department of Cell Biology, Emory University School of Medicine, Atlanta, GA, [3]Department of Oncological Sciences, Hunstman Cancer Institute, University of Utah, Salt Lake City, UT

Nuclear pore complexes (NPCs) mediate communication between the nucleus and cytoplasm in eukaryotic cells and are the major gateways for the diffusion of small molecules, as well as signal-mediated transport of proteins, RNAs and RNPs. The NPC is composed of ~30 different proteins (nucleoporins) and most of them are localized symmetrically on both sides of the NPC, whereas only few nucleoporins localize to either its cytoplasmic or nuclear face. The presence of distinct phenylalanine-glycine (FG) repeats domain is a common feature of many nucleoporins. Most likely, the FG repeat motifs play a critical role for the interaction between the NPC and soluble transport receptors. We have recently mapped the domain topology of the vertebrate nucleoporin Nup214/CAN within the *Xenopus laevis* NPC by using domain-specific antibodies in combination with expression of epitope-tagged Nup214/CAN in *Xenopus laevis* oocytes. By doing so, we could show that this nucleoporin is anchored to the cytoplasmic side of the NPC by its non-FG-repeat domains (i.e. its N-terminal and central domain), whereas its C-terminal FG-repeat region appears mobile within the NPC. Moreover, the localization of the FG-repeat domain correlates with the transport state of the NPC. In this context, an arrest of nuclear transport restricts the FG-repeats of Nup214/CAN to their anchoring site within the NPC. Additionally, the localization of the FG-repeat domain is influenced by excessive import or export cargo, indicating that FG-repeat domains chaperone cargo through the NPC. Cellular calcium and ATP levels affect NPC conformation and nuclear transport. We show now that conformational changes of the NPC induced by $Ca^{2+}$ or ATP have only a minor effect on the mobile character of the FG-repeat domain of Nup214/CAN.

2706

### Characterisation of Emerin-Nesprin Interaction

M. A. Wheeler,[1] Q. Zhang,[2] J. D. Davies,[2] C. M. Shanahan,[2] J. A. Ellis[1]; [1]Randall Division of Cell and Molecular Biophysics, King's College, London, United Kingdom, [2]Department of Medicine, Addenbrooke's Hospital, Cambridge, United Kingdom

X-linked Emery Dreifuss muscular dystrophy (EDMD) is a neuromuscular disease with associated cardiomyopathy and arises due to mutations in the inner nuclear membrane protein emerin. Recently we have identified nesprin-1 and 2 as emerin binding partners and shown that both emerin and the nesprins localise to the nuclear envelope and sarcoplasmic reticulum in cardiac tissue. This work was carried out to characterise the emerin-nesprin interaction and determine whether disruptions to this association contribute to defects observed in EDMD. To identify specific regions in both emerin and nesprin involved in the interaction between these proteins, fragments of nesprin-1α, 2α and 2β including each spectrin-like repeat (SR) were amplified and expressed as GST and $his_6$ tagged fusion proteins. Similarly, emerin constructs covering the entire protein were made. Using blot-overlay assays, GST pull-downs, in vitro transcription/translation co-immunoprecipitation and BIACORE analysis we have established that residues 368 to 919 (LEM-like domain to SR5) of nesprin-1α and 1-219 (SR1 to SR2) of nesprin-2β mediate binding to emerin. Having identified those residues that mediate the interaction between emerin and nesprin we have begun to determine whether emerin mutations found in patients with X-EDMD affect this association. To substantiate these findings we have characterised the subcellular localisation of nesprin and emerin in primary neonatal rat cardiomyocytes. This work will provide further insight into the emerin-nesprin interaction.

2707

### Predetermined Nuclear Pores Mediate Transport of Glucocorticoid-induced Cargos

Y. Ludwig, C. Schäfer, H. Oberleithner, V. Shahin; Institute of Physiology II, University of Münster, Münster (NRW), Germany

Steroid hormone induced gene expression is a vital intracellular process that requires translocation of hormone-receptor-dimers from the cytosol to the chromatin. Nuclear translocation of the several thousands dimers occurs through nuclear pore complexes (NPCs) whose total number exceeds many millions in the nuclear envelope. Hence, the question should be raised, whether each NPC could be non-randomly recruited to translocate hormone-receptor-dimers to their target genes that are in turn located in limited domains of the chromatin. To address this question, we expressed glucocorticoid receptors (GRs) in *X. laevis* oocytes and then stimulated them with a potent agonist, triamcinolone acetonide (TA). Over a time scale of 90 seconds to 24 hours after TA injection, nuclear envelope permeability and structure were investigated by fluorescence and atomic force

microscopy. Passive nuclear envelope permeability for fluorescent macromolecules transiently decreased during import of TA-induced GRs (5.53% after 90 seconds of TA stimulation) and export of newly-synthesized mRNA transcripts (10.35% after 20 minutes of TA stimulation) visualized on the nuclear envelope surface at single molecule level. During transport of GRs and mRNA, local areas of the nuclear envelope surface were found divided into two at least distinct populations of NPCs, transporting or resting ones that alternate with each other. We conclude that steroid hormone signalling to the nucleus occurs at selective sites of the nuclear envelope and involves predetermined NPCs.

2708

**Biophysical Characterization of the Head-To-Tail Interaction of Human Lamin Dimers**

J. Schumacher,[1] L. Kapinos-Schneider,[2] N. Mucke,[3] A. Lustig,[2] S. V. Strelkov,[2] U. Aebi,[2] H. Herrmann[1]; [1]Molecular Genetics, German Cancer Research Center, Heidelberg, Germany, [2]M.E.Mueller Institute, University of Basel, Biozentrum, Basel, Switzerland, [3]Biophysics of Macromolecules, German Cancer Research Center, Heidelberg, Germany

We have recombinantly synthesized the amino- and carboxy-terminal end fragments of the alpha-helical rod domain (12 and 5 heptades, respectively) with varying parts of the flanking non-alpha-helical segments of human lamins A, B1 and B2. These fragments represent regions that were implicated in the head-to-tail association of lamin dimers (Heitlinger et al., J. Cell Biol. 113: 485-495). Hence, we analyzed their sedimentation properties by analytical ultracentrifugation after reconstitution from urea into buffers of different ionic strength. The amino-terminal fragments were soluble and sedimented with distinct profiles and peak values of around 1.9 *S* as expected for coiled-coil dimers. In contrast, the carboxy-terminal fragments were, except for that of lamin B2, largely insoluble and precipitated so that no meaningful *S* value could be determined. Precipitation was partly overcome by increasing the salt concentration that, in turn, yielded symmetrical peaks of around 1.5 *S* for these carboxy-terminal fragments in agreement with their smaller size compared to the amino-terminal fragments. Interestingly, mixing of equimolar amounts of the corresponding amino- and carboxy-terminal rod end fragments before centrifugation gave rise to entirely soluble complexes with *S* values peaking between 2.3 and 2.7 *S*. These *S* values are compatible with an ordered tetrameric association of the amino- and carboxy-terminal fragments, as was further corroborated by sedimentation equilibrium centrifugation analysis. Moreover, we show that this association, mimicking the head-to-tail interaction of lamin dimers, also occurs between the respective heterologous pairs of A- and B-type fragments, suggesting that A-type lamins may associate head-to-tail with B-type lamins in any combination. This finding is of considerable importance concerning the more general concept of how lamins may associate *in situ*, for example, within the nuclear lamina.

2709

**Association of Nup153 with the Nuclear Lamina**

K. J. Roux, P. Enarson, M. Crisp, B. Burke; Anatomy and Cell Biology, University of Florida, Gainesville, FL

The nuclear envelope (NE) consists of two contiguous lipid bilayers that delineate the nucleus from the cytoplasm. Studded with large nuclear pore complexes (NPCs) the NE is composed of multiple protein subunits that span the inner (INM) and outer nuclear membrane (ONM). NPCs facilitate regulated transit of macromolecules between the nucleus and cytosol. With a purported role in protein and RNA trafficking, Nup153 is a highly mobile O-linked nucleoplasmic glycoprotein detected at the NPC basket. Nup153 has three distinct domains, an N-terminal region required for targeting and assembly to the NPC, a central zinc finger motif and a C-terminal domain with multiple FG repeats involved in selective nuclear transport. The N-terminal region of Nup153 (aa 3-144) targets the protein to the surface of the inner nuclear membrane (INM) while aa 39-339 are required for NPC assembly. We hypothesized that the INM targeting motif of Nup153 (N144) may interact with nuclear lamins, the filamentous proteins that constitute the mesh-like lamina adjacent to the INM. Our studies identified the requisite expression of lamins A and C (LaA/C) for the proper targeting of N144 to the NE. Furthermore, exogenous wild type, prelamin A and LaA Δ50 (progerin), but not LaC or the fully processed mature LaA colocalize with N144. These results suggest that, by means of the interaction between Nup153 and immature lamin A, NPCs may nucleate *de novo* lamina assembly.

2710

**Nuclear Transport May be Regulated by Competition Among Karyopherins for the Translocation Channel of the Nuclear Pore Complex**

N. Shulga,[1] L. J. Terry,[2] A. Mason,[1] S. Wente,[2] D. S. Goldfarb[1]; [1]Biology, University of Rochester, Rochester, NY, [2]Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

The nuclear pore complex (NPC) catalyzes the translocation of macromolecules across the nuclear envelope. Cargos are funneled to the NPC by a large family of targeting receptors called karyopherins (Kaps). Kap-cargo complexes bind to 11 phenylalanine-glycine (FG) repeat-containing nucleoporins within or about the central translocation channel. We have been investigating the requirements that different Kaps have for various FG-repeat domains. Based on these studies we already know that not all Kaps are affected identically by the targeted deletion of various FG-repeat domains (Strawn et al., Nat. Cell Biol. 6:197-206 (2004). We are currently testing the hypothesis that nuclear trafficking can be regulated in response to physiological cues by modulating the interaction of certain Kaps with the translocation channel. Kap60p/Kap95p (importin α/β1)-mediated translocation is significantly more resistant than either Kap121p or Kap104p-mediated transport to the deletion of certain FG-domains. Now, we have mapped in some detail which FG-repeat domains are most critical for Kap104p-mediated transport. In some FG-domain deletions, heat shock or over-expression of *SSA1* (Hsp70) both stimulates Kap95p-mediated transport and inhibits Kap104p-mediated transport. Together, these results indicate that Kap95p competes better than Kap104p and Kap121p for the translocation channel, and argue against the alternative hypothesis that Kaps follow physically distinct pathways through the translocation channel. It remains possible that the preferential binding of various Kaps to certain classes of FG-repeat motifs also controls passage through to the translocation channel. We conclude that the nuclear transport of protein and RNA cargos could be regulated in vivo by controlling competition among Kaps for access to FG-repeats within the translocation channel, for example, during heat shock.

2711

**The Lamina of Hutchinson Gilford Progeria Syndrome Cell Nuclei Lacks the Ability to Rearrange**

K. N. Dahl,[1] P. Scaffidi,[2] D. E. Discher,[3] T. Misteli,[2] K. L. Wilson[1]; [1]Department of Cell Biology, Johns Hopkins School of Medicine, Baltimore, MD, [2]National Cancer Institute, NIH, Bethesda, MD, [3]Department of Chemical and Biomolecular Engineering, University of Pennsylvania,

Philadelphia, PA
The premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS) is caused by mutations in *LMNA*, the gene encoding A-type lamins. Lamins polymerize to form a network of structural filaments at the nuclear envelope and throughout the nucleus, with hypothesized roles in the mechanical stability of the nucleus. Both mechanical stability and mechano-sensitive gene expression are defective in *lmna*-null cells, but the underlying nature of the mechanical properties of the lamina are poorly understood. In this study, micropipette aspiration was used to quantify the viscoelastic properties of isolated, somatic nuclei. The effects and distribution of the applied forces were visualized using GFP-lamin B and Hoechst 33342 stained chromatin. To experimentally separate the mechanical contributions of major nuclear elements (chromatin or lamina), nuclei were also pre-swelled using different concentrations of salt. At high salt, chromatin condenses and is the primary force-bearing element. With low salt the lamina of the nucleus sustains much of the load. The mechanical properties of nuclei from a variety of cells, including those from HGPS patients, were measured. Nuclei from HGPS patients show significant reduction in the ability to rearrange the lamina under mechanical stress and deformation in both the salt swelling/shrinking and micropipette aspiration assays. In contrast to normal nuclei, which 'buckle' homogeneously in response to mechanical stress, HGPS nuclei collapsed along major axes, suggesting catastrophic failure to distribute applied forces across the entire lamina. When visualized using crossed polarizers wildtype nuclei appeared dark, consistent with a randomly-oriented lamina network. However HGPS nuclei were strikingly birefringent suggesting the HGPS lamin network has locally-ordered microdomains. These microdomains can explain most of the mechanical differences seen here between HGPS and normal cells, and may have functional consequences in disease.

2712

**Targeting Protein for Xklp2 (TPX2) is Required for Nuclear Expansion in Xenopus Egg Extracts**

L. O'Brien,[1] L. Wells,[2] C. Wiese[1]; [1]Dept. of Biochemistry, UW-Madison, Madison, WI, [2]Complex Carbohydrate Research Center, University of Georgia, Athens, GA
The Targeting Protein for Xklp2 (TPX2) is necessary for mitotic spindle assembly, but it localizes to the nucleus during interphase (Wittmann et. al., 2000. JCB 149:1405). Mass spectrometry analysis of TPX2 interacting proteins revealed that Lamin associated polypeptide 2 (LAP2), a lamin and chromatin binding protein important for nuclear assembly, is a potential binding partner of TPX2. Consistent with this, purified LAP2 and TPX2 interacted *in vitro* and these proteins could be co-immunoprecipitated from interphase extracts. To determine whether LAP2 binding reflects a role for TPX2 in the nucleus, we depleted TPX2 from interphase *Xenopus* egg extracts and induced the extracts to assemble nuclei. Although nuclei formed in TPX2-depleted extracts possessed nuclear membranes, a lamina and nuclear pore complexes, and they were able to import rhodamine-labeled nucleoplasmin and to exclude 155 kD dextran, these nuclei were significantly smaller than those assembled in mock depleted extracts. Immunofluorescence staining showed that LAP2 localized to the nuclear rim in mock-depleted nuclei but was diffusely distributed in TPX2-depleted nuclei, suggesting that TPX2 may be involved in targeting LAP2 to the nuclear rim. Our data further suggest that the interaction between TPX2 and LAP2 may be required to allow nuclei to grow.

2713

**Barrier-to-Autointegration Factor (BAF) Accumulates in the Nucleus During S Phase and Its Loss Delays Progression of S Phase in HeLa Cells**

T. Haraguchi,[1] T. Koujin,[1] T. Kojidani,[1] H. Osakada,[1] C. Mori,[1] K. L. Wilson,[2] Y. Hiraoka[1]; [1]CREST Research Project, Kansai Advanced Research Center, Kobe, Japan, [2]Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD
Barrier-to-autointegration factor (BAF), a DNA-bridging protein, is essential for embryonic development in *C. elegans* and *Drosophila*. BAF has roles in chromosome condensation/decondensation, nuclear assembly, and gene expression, including cyclin gene expression in *Drosophila*, but its functions are poorly understood in mammalian cells. We examined the subcellular localization of BAF by indirect immunofluorescence staining using a specific antibody for endogenous BAF and HA-tagged BAF in various human cell lines. The cells studied included both mortal (TIG1, and WI-38) and transformed/immortalized lines from various tissues (e.g., A431, SiHa, and HeLa). In interphase cells, BAF localization varied both within and between cell types. Interestingly, BAF localized mainly in the nucleus in some cells, but the cytoplasm of others, suggesting its interphase localization is dynamically regulated during the cell cycle, and perhaps further regulated by cell type. In TIG1 cells, which are mortal, the fraction of cells with predominantly nuclear-localized BAF ('nucleus-positive') decreased upon aging, suggesting BAF is involved in cell proliferation or aging events. Consistent with proliferative roles, studies with immortal cell lines including HeLa cells showed that ~ 30% of non-synchronized cells were nucleus-positive for BAF, whereas ~80% of S-phase-synchronized cells were nucleus-positive. Independently supporting this result, ~80% of actively-replicating (BrdU positive) cells in non-synchronized populations were nucleus-positive for BAF. These results suggested that BAF localizes predominantly in the nucleus during S phase. To test this idea, HeLa cells were subjected to RNAi to downregulate BAF expression. In BAF-RNAi cells the fraction of BrdU-positive cells was 50%, compared to 30% of nonspecific-RNAi controls, indicating that S-phase was prolonged by loss of BAF. These results suggest that BAF concentration in the nucleus is required for normal progression through S-phase in human cells.

2714

**Dynamics and Ultrastructure of the Reforming Nuclear Envelope at Telophase as Revealed by Time-lapse, FRAP, FRET and EM Analyses**

T. Shimi,[1] T. Koujin,[1] T. Kojidani,[1] A. Yamamoto,[2] Y. Hiraoka,[1] T. Haraguchi[1]; [1]CREST Research Project, Kansai Advanced Research Center, Kobe, Japan, [2]Nagahama-Bio University, Nagahama, Japan
Reformation of the nuclear envelope (NE) during mitotic telophase is essential for higher eukaryotic cells to re-establish functional nuclear structures. To understand early (telophase) events of NE assembly, we examined the localization, mobility, and molecular interactions of NE proteins in living cells using fusions with GFP variants. We particularly focused on BAF, a DNA-binding protein that recruits nuclear membrane proteins (e.g., emerin) and A-type lamins to reforming nuclei. In human cells, BAF, lamin A, emerin and other 'LEM-domain' proteins (MAN1, LAP2beta) all localize in a specialized structure close to the central region of a telophase chromosome mass, designated the "core region", when the NE is reforming at telophase. Previous FRAP analysis showed that BAF is highly mobile, whereas lamin A is immobile in the interphase NE.

In contrast, BAF and lamin A as well as emerin, MAN1 and LAP2beta all have similarly low mobility in the core region, suggesting they co-assemble stable structure(s) in the core region. iFRET and time-lapse FRET analyses of the core region showed BAF associates very tightly with other BAF molecules (very high level of FRET with BAF), and also associates, albeit more weakly with emerin (low but detectable FRET). These results show that BAF directly interacts with itself and emerin in the core region. To further examine the re-forming nuclear envelope in high resolution, we combined live observation with EM observation, and found that BAF formed an electron-dense structure at the surface of the core region. We conclude that BAF is organizes as a solid immobile structure in the core region, which recruits emerin and lamin A. We hypothesize that formation of this stable BAF complex is a key step in re-forming a functional nucleus.

2715

**Flexible Nucleoporins as Entropic Barriers to Nucleocytoplasmic Transport**

R. Y. H. Lim,[1] J. Köser,[1] N. Huang,[1] K. Schwarz-Herion,[1] J. Deng,[2] A. K. H. Lau,[2] B. Fahrenkrog,[1] U. Aebi[1]; [1]M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland, [2]Institute of Materials Research and Engineering (IMRE), Singapore, Singapore

Natively unfolded phenylalanine-glycine (FG)-repeat domains are alleged to form the physical constituents of the selective gate in nuclear pore complexes (NPCs) during nucleocytoplasmic transport. At present, several key aspects of the barrier remain elusive and experimentally unsubstantiated. Examples include the conformational make-up of the FG-repeat domains, the simultaneous trapping mechanism of the barrier, and how these characteristics contribute to the overall kinetics of nucleocytoplasmic transport. To provide insight into the gating mechanism, an experimental platform which mimics the general physical dimensions of NPCs has been constructed to investigate the behavior of surface-tethered, FG-repeat domains at the nanoscale. Atomic force microscope force measurements indicate that the collective behavior of such FG-repeat domains gives rise to a long-range, exponentially decaying repulsive force in physiological buffer. This force acts as a barrier which can effectively repel oncoming passive cargoes from the NPC near-field. The measured force is appropriately described by the Alexander-de Gennes scaling theory which indicates that the molecules are thermally mobile and exist in an extended polymer brush-like conformation. This assertion is confirmed by observing that the brush-like conformation undergoes a reversible collapse transition by increasing the hydrophobic content in the buffer. Importantly, this finding does not preclude the fact that the entropic barrier can also serve to “trap” cargoes containing nuclear localization signals (NLS) in the near-field of an NPC via specific binding interactions between transport receptors (e.g. Karyopherin/Importin) and FG-sites. These results reveal how FG-repeat domains may simultaneously function as an entropic barrier and a selective trap in the near-field of native NPCs i.e. selective gate.

2716

**Nesprins and Sun Domain Proteins Link the Actin Cytoskeleton to the Nuclear Lamina**

M. Crisp,[1] Q. Liu,[1] K. J. Roux,[1] B. Burke,[1] P. D. Stahl,[2] D. Hodzic[2]; [1]Anatomy and Cell Biology, University of Florida, Gainesville, FL, [2]Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO

Nesprins and SUN domain proteins link the actin cytoskeleton to the nuclear lamina The nuclear envelope (NE) defines the barrier between the nucleus and cytoplasm. Its major features are the inner and outer nuclear membranes (INM and ONM) separated by a 50-100nm perinuclear space (PNS). The mammalian INM contains roughly 50 unique polypeptides. Among these, Sun1 and Sun2, a pair of type II integral membrane proteins, are characterized by an evolutionarily-conserved C-terminal stretch of 200 amino acids (SUN domain) that localize in the perinuclear space. Sun proteins are anchored in the INM via their nucleoplasmic N-terminal domains which interact with components of the nuclear lamina (NL). The NL is a major structural element of the NE that is closely associated with the nuclear face of the INM and is composed primarily of A- and B-type lamins. In the case of Sun2, its localization within the INM is partially dependent upon the presence of A-type lamins. While the ONM is biochemically related to the ER, it does contain certain unique proteins. One of these is nesprin2 giant, an 800kDa type II membrane protein that contains a small C-terminal lumenal domain and a giant cytoplasmic domain featuring multiple spectrin repeats and an N-terminal actin binding region. Localization of nesprin2 giant is dependent upon the Sun proteins since co-depletion of Sun1 and 2 by RNAi leads to loss of ONM-associated nesprin. Furthermore, overexpression of a soluble form of the Sun1 lumenal domain leads to a similar loss of nesprin2 giant from the ONM. Cells subjected to either treatment exhibit dilation of the ONM. Our data indicate that Sun1/2 and nesprins interact across the PNS. In this way these proteins form a molecular linkage between the actin cytoskeleton and the NL.

2717

**Sorting of Membrane Proteins to the INM: Protein-protein Interactions Occurring during this Process**

S. C. Braunagel; Entomology, Texas A&M University, College Station, TX

A short sequence of 33 amino acids derived from an envelope protein of the occlusion derived virus of baculovirus is sufficient to direct fusion-proteins to the viral envelope which assembles in the nucleus; and in the absence of infection, direct fusion-proteins to the inner nuclear membrane (INM). This sequence has been named a viral INM sorting motif. Using the viral sorting motif as probe, chemical crosslinking studies designed to covalently crosslink temporal intermediates in the trafficking pathway suggest that during infection, specific protein-protein interactions regulate trafficking from the ER to the INM: sorting begins at the translocon; and during transit from the ER to INM multiple protein-protein interactions are detected. A putative cellular cognate of a viral protein that crosslinks to the INM-sorting motif has been identified and has been named SMAP-10 (sorting motif associated protein of 10 kDa). Directed studies of SMAP-10 show that like its viral counterpart, it is membrane associated, present within the ER and likely functions as a cellular sorting factor to facilitate transport of integral membrane proteins to the INM. These data suggest that for some integral membrane proteins, the mechanism of protein trafficking to the INM involves multiple sorting events; and the study of the trafficking of baculovirus envelope proteins represents a viable model system to identify and expand our understanding of the molecular basis of protein sorting to the INM.

2718

**Study of the Nuclear Pore Complex Nup107-160 in Interphase and Mitosis**

M. Platani; Gene Expression Unit, European Molecular Biology Laboratory, Heidelberg, Germany

Fellow In eukaryotes, trafficking between the nucleus and the cytoplasm occurs via large multiprotein structures inserted in the nuclear envelope

(NE), the nuclear pore complexes (NPC). At the beginning of mitosis the nuclear envelope and the NPC break down. NPC disassembly is initiated by direct phosphorylation of a subset of nucleoporins via the mitotic cdc2/cylcin B kinase [1]. Reformation of the nuclear envelope at the end of mitosis involves early association of a subset of nucleoporins (the Nup107-160 complex) with chromatin followed by association of nuclear envelope precursor membranes. The Nup107-160 complex, composed of nine nucleoporins, has been demonstrated to play a critical role in postmitotic NPC formation since its absence results in nuclei with nuclear envelope devoid of NPCs [2,3]. To further characterize the mechanism of the temporal and spatial regulation of the Nup107-160 complex in NPC reassembly around chromatin, we are using the *in vitro Xenopus* system for biochemical studies and mammalian HeLa cells for high resolution live cell imaging. We generated antibodies against three of the nine components of the complex the Nup43, Nup37 and seh1. Preliminary data do suggest differences in the phosphorylation state of the complex between interphase and mitosis. Combining biochemical analysis and RNAi we are trying to understand how the modification of the complex is achieved and how this affects its interaction with chromatin and final assembly into a functional NPC. 1. Suntharalingam, M. et al. Peering through the Pore: Nuclear Pore Complex Structure Assembly , and Function. *Developmental Cell* **4,** 775-789 (2003).

2719

**Sequence Preference in RNA Recognition by the Nucleoporin Nup153**

A. E. Bilak, J. Ball, C. Dimaano, K. Ullman; Oncological Sciences, Huntsman Cancer Institute, Salt Lake City, UT

Nuclear pore complexes bridge the inner and outer nuclear membrane to form aqueous channels through which traffic between the cytoplasm and nucleus takes place. These macromolecular structures are composed of multiple copies of ~30 different proteins that work coordinately to guide cargo-receptor complexes in transit. Interactions between nuclear pore proteins and transport receptors facilitate movement through the pore, although in some cases nuclear pore proteins contact cargo directly. In exploring the range of interactions that are possible at the pore complex, we previously found that the pore protein Nup153 can bind RNA directly. The ability to bind RNA was consequently mapped to 150 amino acids. This novel RNA binding region was found to bind to several different mRNAs, but not to tRNA, snRNA, or 5S rRNA, when tested in an in vitro pull-down assay. Here, we have further characterized the RNA recognition properties of this nuclear pore protein. We first used RNaseH-directed cleavage to determine if there was a dominant binding sequence within a short, plasmid-derived RNA that is recognized by the Nup153 RNA binding domain. We found that these shorter RNA fragments reveal distinct binding preferences. While the molecular determinants of recognition have yet to be fully defined, our results to date indicate that nucleotide bias (toward cytidine and guanosine) contributes to recognition. However, nucleotide content did not strictly correlate with binding ability. Using synthetic RNA oligonucleotides, we tested and confirmed that sequence arrangement is important as well. Given the previous observation that the Nup153 RNA binding domain can associate with a spectrum of mRNAs, our working model is that an RNA motif with a fair degree of flexibility allows Nup153 to discriminate between different classes of RNA and may mediate direct contact during the process of mRNA export.

2720

**Impact of Karmellae on Nuclear Organization and Inheritance in S cerevisiae**

T. Hattier, A. M. Tartakoff; Pathology, Case Western Reserve University, Cleveland, OH

Overexpression of a number of individual transmembrane proteins of the ER results in the production of “karmellae,” which are compatible with ongoing cell growth. As first described by R. Wright and J. Rine in their studies of HMG CoA-Reductase I overexpression (JCB, 107, 101-114 (1998)), these multilayered structures often surround more than half of the perimeter of the nucleus. The characteristic myelin-like units are highly enriched in the overexpressed protein and lack nuclear pores (NPC). As a result, the nucleus becomes anatomically and functionally polarized, with import and export being restricted to only a fraction of the perimeter. By construction of strains in which individual proteins are tagged with GFP or RFP, we observe that the nucleolus associates closely with karmellae, while the spindle pole body does not. Lac operator-tagged centromeric and telomeric loci do not show an obvious preference for either portion of the nuclear perimeter. Selected electron micrographs of Wright and Rine show that karmellae are retained by the mother cell and do not enter the bud. We have confirmed these observations by examining cells in anaphase which express GFP-tagged HMG CoA-Reductase. Since the septin ring has been implicated in segregation of cortical proteins between mother and bud, we have also studied tagged karmellae in septin mutants. Restriction of these structures is not altered upon inactivation of individual septins. Similar behavior is characteristic of the ESCapades which are produced upon accumulation of the lamin-like protein, Esc1p (see accompanying poster/talk). We therefore suggest that their lack of inheritance - rather than resulting from the presence of a physical barrier - reflects the existence of a coherent “maternal domain” of the nuclear perimeter.

2721

**Functional Analysis of C38D43, an AT-hook Protein Implicated in Nuclear Pore Function and Chromosome Segregation in Caenorhabditis elegans**

A. G. Fernandez, F. Piano; Biology, New York University, New York, NY

The advent of functional genomics has enhanced the potential for specific functional prediction of genes. Using an RNAi-based approach, several studies of the early *C. elegans* embryo have revealed detailed phenotypes for hundreds of genes required for embryogenesis. Through the systematic analyses of discrete characters in the early embryo and the generation of a phenotypic signature based on a digital representation of these characters, we developed phenoclustering, a means of grouping genes based on phenotype. Using this technique, a novel AT-hook domain protein C38D4.3 was grouped in one case with genes known to be required for chromosome separation (Piano et. al, 2002) and in another study with the nucleoporins (Sönnichsen et al., 2005 ). *In vivo* localization of C38D4.3 showed that it shuttles between the kinetochore and the nuclear periphery during the cell cycle. To test the hypothesis that this gene is required for chromosome segregation, we have analyzed a histone:GFP fusion in embryos RNAi-depleted of C38D4.3 and observed chromatin bridges at anaphase. We are now studying this gene by analyzing how its localization changes when genes with similar phenotypic signatures are depleted using RNAi. Using this approach we have identified *npp-9*, the *C. elegans* homologue of RanBP2, as a gene required for the proper nuclear envelope localization of C38D4.3. RanBP2 is an effector of the small GTPase Ran that is required for nucleocytoplasmic transport and postmitotic nuclear envelope re-formation. Thus our observations are consistent with the idea that C38D4.3 functions in both chromosome segregation and the nuclear envelope, providing support for both hypotheses generated by phenoclustering.

## Invertebrate Development (2722-2734)

2722

### Echinoid Mosaic Epithelia as a Model for Developmental Boundaries in Drosophila

C. E. Laplante, L. A. Nilson; Biology, McGill University, Montreal, PQ, Canada

Smooth boundaries between cell populations can reflect sorting based on affinity differences, but the causes of these differences and the subcellular structure of the boundary remain obscure. The *Drosophila* ovarian follicular epithelium is a simple model system that allows the generation of ectopic boundaries through the production of genetic mosaics. In follicular epithelia mosaic for the *echinoid*$^{F72}$ (*ed*$^{F72}$) allele, an actomyosin ring assembles at the clone border, constricting the clones apically. Interestingly, levels of DE-Cadherin and β-catenin are reduced at the interface between *ed*$^{F72}$ mutant and non-mutant cells, indicating a destabilization of adherens junctions at the clone border. While this boundary is reminiscent of multicellular contractile rings that assemble during *Drosophila* embryonic dorsal closure, wound healing and apoptotic cell extrusion, we have found no evidence of Jun kinase activity at the *ed*$^{F72}$ clone border and the *ed*$^{F72}$ mutant cells are not apoptotic. During mid-oogenesis *ed*$^{F72}$ mutant clones are indistinguishable from wild type cells, suggesting that *ed* may be temporally regulated. Correspondingly, Ed levels are high during early and late oogenesis but are greatly reduced during mid-oogenesis suggesting that the actomyosin ring between mutant and wild type cells disassembles in the absence of an Ed expression boundary. After mid-oogenesis, Ed first reappears in a dorsal-anterior subset of follicle cells, generating an endogenous Ed expression boundary with the neighboring cells. Interestingly, this localization corresponds to a smooth morphological boundary between two cell populations that will ultimately cooperate to form an epithelial tube. This work implicates Ed in a cell sorting process in the *Drosophila* follicular epithelium, and suggests that the local formation of a contractile actomyosin structure and regulation of adherens junctions may represent a general cell sorting mechanism applicable to developmental boundaries.

2723

### Individual Nuclei in the Drosophila Syncytial Blastoderm Embryo Possess an Organized and Restricted ER/Golgi Membrane System in the Absence of Plasma Membrane Boundaries

M. Mavrakis,[1] D. Frescas,[1] R. DeLotto,[2] J. Lippincott-Schwartz[1]; [1]NIH, Bethesda, MD, [2]University of Copenhagen, Copenhagen, Denmark

Protein secretion within all eukaryotic cells is controlled by the endoplasmic reticulum (ER) and Golgi apparatus, whose spatial organization and dynamics are closely associated with the nucleus and centrosomally-derived microtubules. The Drosophila syncytial blastoderm, a multi-nucleated single cell embryo, initially lacks this organization- with nuclei and centrosome-derived microtubules localized deep within the embryo interior, and maternally-derived ER and Golgi distributed at the embryo cortex. Here, we use GFP reporters, live cell imaging and biophysical techniques to investigate how ER and Golgi structures become associated with nuclei in the syncytial blastoderm, and how the ER and Golgi become equally apportioned into separate cells at cellularization. When nuclei were still localized in the embryo interior, we found that cortical ER existed as a single interconnected membrane system within which resident ER components rapidly diffused. The Golgi apparatus was closely associated with the cortical ER and consisted of thousands of small structures containing proteins undergoing constitutive cycling into and out of the ER. Upon nuclear arrival at the cortex, the lateral diffusion and inter-organellar movement of ER and Golgi components became restricted to ER and Golgi membranes surrounding individual nuclei, resulting in each nucleus being surrounded by a distinct ER and Golgi membrane system in the absence of a plasma membrane boundary. This unexpected endomembrane organization within the embryo could serve to ensure that equivalent amounts of ER and Golgi are packaged with nuclei at cellularization. It may further allow secretory products to be released in a spatially and temporally controlled fashion across the embryo both before and during cellularization.

2724

### Drosophila Myosin V

A. Hong, J. Toth, J. R. Sellers; NHLBI, NIH, Bethesda, MD

Class V myosins have been studied extensively in the metazoans and are known to be involved in a wide range of cellular functions. In addition, multiple cargoes of Myosin V have been identified. However, little is known about the functions of the Drosophila myosin V. Our lab is investigating the role of Drosophila myosin V and its potential binding partners by studying the localizations of myosin V in the embryo, larva and adult flies. In the embryo, myosin V is expressed in the neuroblasts in the central nervous system. In the larva, the protein is detected in the salivary gland, imaginal rings, proventriculus, gastric caeca, the digestive system and the Malphigian tubules. The staining of myosin V is punctuated and in some cells it is localized subcortically, suggesting a possibility of its association with other vesicles. Myosin V is strongly expressed in the third instar larva in the imaginal rings. These cells are precursors of the adult tissues and this suggests a correlation between the lethal phase of Myosin V mutants that occurs during the third instar larva. We are examining the role of Myosin V in the transition from a larva to a pupa and adult. From the yeast 2-hybrid analysis, we have a few potential binding partners of Myosin V and we are performing biochemical and genetic interaction studies.

2725

### Biological Mechanisms of Sex Determination and Sex-specific Gene Regulation: Biochemical Studies of Doublesex

W. Zhang, B. li, Y. Yang, J. R. Bayrer, M. A. Weiss; Biochemistry, Case Western Reserve University, Cleveland, OH

*Doublesex (dsx)* is a transcription factor in *Drosophila* that regulates somatic sexual differentiation. Male- and female-specific splicing isoforms of DSX share a novel DNA-binding domain (the DM motif), broadly conserved among metazoan sex-determining pathways. Mutations or deletions of these genes are associated with intersexual abnormalities, including the human birth defects termed “9p syndrome”. Here, we characterize the DM motif by site-directed and random mutagenesis using a yeast one-hybrid system and extend this analysis by chemogenetic complementation in vitro. Mutations that impair DNA binding tend to occur at conserved positions whereas neutral substitutions occur at non-conserved sites. Evidence for a specific salt bridge between a conserved lysine and the DNA backbone is obtained through the synthesis of non-standard protein- and DNA analogs. Second, structure-function relationships in the female-specific C-terminal dimerization domain were investigated by characterizing the sex-specific interaction between DSX$^{F}$ and its putative transcriptional coactivator IX. Our data suggest that the dimerization domain instead of the sex-specific tail is responsible for IX binding. Finally, to elucidate the mechanism by which of DSX$^{F}$ controls female sex-specific behavior, we

have identified two temperature-sensitive mutations in the $DSX^F$ C-terminal dimerization domain. Allelic replacement of wild-type *dsx* by our designed temperature-sensitive alleles promises to provide definitive evidence for the functions of $DSX^F$ in somatic sexual differentiation and courtship behavior. Together, these results provide molecular links between the structure of $DSX^F$ and its function in the regulation of sexual dimorphism.

2726

**The *Drosophila* Dnase II Enzyme Is Required for Hemocyte Immune Function**

C. Seong, R. Ramirez, R. J. Aguilera; Biology, University of Texas at El Paso, El Paso, TX

DNase II enzymes are highly conserved proteins that are required for the degradation of DNA within phagocytes. Engulfment of apoptotic cells and/or bacteria by phagocytic cells requires the function of this nuclease to completely destroy ingested DNA within phagolysosomes. Mutation of the *dnase II* gene results in an increase of undegraded apoptotic DNA within phagocytic cells in mice, nematodes (*C. elegans)*, and fruit flies (*Drosophila melanogaster*). Reduction of DNase II enzymatic activity in *Drosophila dnase Il* hypomorphic mutants results in increased accumulation of DNA in the ovaries with no other obvious phenotypic abnormalities. Due to the importance of DNA clearance after bacterial infection, we hypothesized that a severe reduction of DNase II activity should result in an inability of mutant animals to mount an efficient immune response. To test this hypothesis, we have generated RNAi-*dnase II* transgenic flies. As expected, expression of the RNAi-*dnase II* construct in hemocytes and in whole animals resulted in a severe reduction of DNase II activity and a significant decrease in hemocytes. Furthermore, infection of RNAi-*dnase II* flies with gram negative and positive bacteria resulted in a severe reduction in fly viability. These results confirm that DNase II is essential for hemocyte function and implicate this enzyme as an important component of the immune system of *D. melanogaster*.

2727

**Inhibition of Apoptosis by p26: Implications for Small Heat Shock Protein Function during Development of *Artemia franciscana* Embryos**

T. S. Villeneuve,[1] X. Ma,[2] Y. Sun,[1] M. M. Oulton,[1] A. E. Oliver,[2] T. H. MacRae[1]; [1]Biology, Dalhousie University, Halifax, NS, Canada, [2]Center for Biostabilization, University of California, Davis, CA

p26, an abundantly expressed small heat shock protein, is thought to establish stress resistance in oviparously developing embryos of the crustacean, *Artemia franciscana*, by preventing irreversible protein denaturation, but this molecular chaperone may also promote survival by inhibiting apoptosis. To test this possibility stably transfected mammalian cells producing p26 were generated and their ability to resist apoptosis was determined. Examination of immunofluorescently stained transfected 293H cells by confocal microscopy demonstrated p26 is diffusely distributed in the cytoplasm with a minor amount in nuclei, and as shown by immunoprobing of western blots, p26 constituted approximately 0.6% of soluble cell protein. p26 localization and quantity were unchanged during prolonged culture and the protein had no apparent ill effects on transfected cells. Molecular sieve chromatography in Sepharose 6B revealed p26 oligomers composed of at least 20 monomers, with a second fraction occurring as larger aggregates. A similar pattern was observed in sucrose gradients but overall oligomer size was smaller. Mammalian cells containing p26 were more thermotolerant than cells transfected with expression vector only, and as measured by annexin V labeling, Hoescht 33342 nuclear staining and pro-caspase-3 activation, transfected cells resisted heat- and staurosporine-induced apoptosis more effectively than control cells. These properties of p26 are potentially important to *Artemia* embryos because they are frequently exposed to high temperature in their natural habitat. p26 also blocked apoptosis in transfected cells during drying and rehydration, conditions thought to activate diapause embryos. The findings demonstrate that in addition to functioning as a molecular chaperone, p26 inhibits apoptosis. This is an activity shared by other small heat shock proteins and it has the potential to play an important protective role during *Artemia* development.

2728

**Synthesis and Function of ArHsp21 and ArHsp22, Small Heat Shock Proteins from *Artemia franciscana***

Z. Qiu, T. H. MacRae; Biology, Dalhousie University, Halifax, NS, Canada

Oviparously developing embryos of *Artemia franciscana* are released from females as encysted gastrulae (cysts) which enter diapause, a profound state of metabolic dormancy characterized by extreme resistance to stress. Cysts withstand environmental insults such as high temperature, desiccation, and long term anoxia, with their protection thought to depend upon molecular chaperones. Screening of a cDNA library generated by subtractive hybridization identified two small heat shock proteins in oviparous embryos, these in addition to p26, a thoroughly described member of this chaperone group found in *Artemia*. Full length cDNA sequence was obtained for each small heat shock protein using 3-' and 5'- RACE, and probing of Southern blots with labeled cDNA indicated a single gene for each protein. The small heat shock proteins, named ArHsp21 and ArHsp22, are 29% and 39% identical to p26 in amino acid sequence and they form oligomers, as is characteristic of these proteins. ArHsp21 and ArHsp22 prevented citrate synthase denaturation at 43 °C and the aggregation of insulin under reducing conditions at room temperature, standard *in vitro* assays for chaperone activity. Real time PCR and immunoprobing of western blots demonstrated these small heat shock proteins are synthesized predominately in oviparously developing embryos, with neither detectable by the time larvae reach second instar. After a one hour heat shock at 39 °C small amounts of ArHsp22, but not ArHsp21, were synthesized in adult *Artemia*. ArHsp21 and ArHsp22 have the ability to confer stress resistance during oviparous development of *Artemia* embryos, and ArHsp22 also offers protection to mature animals encountering unfavorable environmental conditions.

2729

**Characterization of p8, a Developmentally Regulated Co-transcription Factor from *Artemia franciscana* Embryos**

Z. Qiu, T. H. MacRae; Biology, Dalhousie University, Halifax, NS, Canada

Embryos of the brine shrimp *Artemia franciscana* undergo either oviparous or ovoviviparous development, with pathway selection dependent on genetic and environmental factors. Ovoviviparous development results in release of swimming larvae from females. In the oviparous pathway, however, development arrests at gastrulation, encystment pertains and metabolism shuts down, a complex process undoubtedly subject to several regulatory mechanisms. Screening of a library produced by subtractive hybridization yielded a partial cDNA from oviparous *Artemia* embryos for the co-transcription factor p8, a protein known to influence cell growth in other organisms. Full length p8 cDNA sequence was obtained by 3'- and 5'-RACE, yielding a deduced amino acid sequence 55% and 66% identical to human and *Drosophila* p8, respectively. Probing of restriction

digested genomic DNA on Southern blots with labeled cDNA indicated a single p8 gene in *Artemia.* As demonstrated by 5'-RACE the p8 gene has two transcription start sites, both of which are active, but proteins of only one size are produced implying a single translation start site. Approximately one kb of putative upstream regulatory region was obtained by LA PCR *In Vitro* Cloning technology and sequence analysis identified several potential transcription factor binding sites. Real time RT-PCR showed that p8 mRNA was restricted almost entirely to the oviparous pathway, peaking in amount at one day post-fertilization and then declining gradually during encystment. The synthesis and localization of p8 in *Artemia* embryos will be further analyzed by application of immunological procedures using available antibodies. p8 has the potential to influence *Artemia* embryo development, perhaps by aiding in the selection of expressed genes as oviparous development initiates.

2730

**The Identification of Microtubule-Associated Proteins during Development of the Brine Shrimp, *Artemia franciscana***

P. A. O'Connell,[1] D. M. Pinto,[2] K. A. Chisholm,[2] T. H. MacRae[1]; [1]Biology, Dalhousie University, Halifax, NS, Canada, [2]National Research Council Institute for Marine Biosciences, Halifax, NS, Canada

Microtubules are dynamic polymers that influence cell shape, polarity, and movement of intracellular components. The organization and function of microtubules are determined by microtubule-associated proteins (MAPs) which associate with these cytoskeletal elements *in vivo* and co-purify with tubulin *in vitro.* This study represents the first large scale identification of MAPs in post-diapause cysts of the crustacean *Artemia franciscana*, where MAPs are defined as salt-extractable proteins which co-purify with microtubules upon taxol-induced tubulin assembly and centrifugation through sucrose cushions. MAPs obtained from encysted *Artemia* after 0, 6, and 12 hours of development were resolved by two-dimensional gel electrophoresis. One hundred and ninety three MAPs, 73 from undeveloped cysts, and 60 each from cysts developed 6 and 12 hours, were selected for identification by mass spectrometry. High confidence peptide identity data ($p<0.05$) were obtained for 85 of the *Artemia* MAPs, and fifteen occurred in at least 2 stages of development. The identified MAPs were grouped according to function. For example, molecular chaperones were identified and they likely arise as part of a stress response in which tubulin and other proteins are protected as embryos encyst and enter diapause. The observation of several ribosomal proteins and elongation factors suggests encysted embryos are primed to synthesize proteins once development resumes and the association of metabolic enzymes with microtubules may facilitate the flow of intermediates through metabolic pathways, thus promoting growth. The study has revealed many interesting MAPs within *Artemia* embryos, indicating how protein-protein interactions within the cytoskeleton contribute to development and growth.

2731

**Detection of Lectin from Banana Prawn (*Penaeus merguiensis*) by Hemagglutination and ELISA**

W. Rittidach,[1] P. Utarabhand,[2] J. L. Hedrick[1]; [1]Animal Science, University of California, Davis, CA, [2]Biochemistry, Prince of Songkla University, Hatyai, Thailand

Lectins in the hemolymph of invertebrates are active participants in cellular and humoral immune recognition mechanisms. The quantification of lectin in shrimp hemolymph is essential to evaluate shrimp's resistance to pathogenic bacteria. In our studies, serum lectin of *Penaeus merguiensis* contained high hemagglutinating activity against rabbit red blood cells. Hemagglutination was inhibited by *N*-acetyl neuraminic acid and fetuin. We purified lectin from *Penaeus merguiensis* hemolymph by affinity chromatography on a fetuin-agarose column and subsequently by gel filtration chromatography using a Superose 12 column. The molecular mass of the lectin was approximately 316.2 kDa. Purified lectin shows two bands (32.3 and 30.9 kDa) on SDS-PAGE with similar isoelectric points of 6.0. The amino acid composition of the purified lectin was determined on a Waters Picotag system. Purified lectin is mainly composed of hydrophobic and polar amino acids. An amount of the lectin antibody was raised by the purified lectin in the albino male rabbit. The serum IgG fraction was partially purified by ammonium sulfate precipitation and DEAE-Sephacel column. The partially purified antibody reacted with the purified lectin by a Western blot of the hemolymph. The antibody was used to develop ELISA (Enzyme-Linked Immunosorbent Assay). The ELISA standard curve was linear over a range of 0.17-2.33 ng/µl lectin. The level of hemolymph lectin was determined by means of ELISA and hemagglutination. Since the lectin level and hemagglutination activity in shrimp hemolymph changed in different stages of oogenesis, the hemolymph lectin may be involved in oogenesis of *Penaeus merguiensis.*

2732

**Differential Effect of Illumination on *in hospite* and Isolated Zooxanthellae: Identification of the 36 kDa Protein and its Role in Cnidarian-dinoflagellate Endosymbiosis**

C. Chen,[1] H. Huang,[1] T. Yeh,[1] H. Huang,[2] W. Uang,[1] L. Fang[3]; [1]Institute of Marine Biotechnology, National Dong Hwa University, Checheng, Pingtung, Taiwan Republic of China, [2]Research and Planning, National Museum of Marine Biology & Aquarium, Checheng, Pingtung, Taiwan Republic of China, [3]Institute of Marine Biodiversity and Evolution, National Dong Hwa University, Checheng, Pingtung, Taiwan Republic of China

Cnidarian-dinoflagellate endosymbiosis represents an important cellular regulation in various marine invertebrates, such as reef-building corals and anemones. The symbiont dinoflagellate (e.g. the *Symbiodinium* spp., or zooxanthellae in generic name) reside in endoderm cells of host animals, which initiates a dynamic inter-transportation of nutrition and a series of complex cell-cell interaction. Nevertheless, molecular mechanism of this regulation to maintain the endosymbiotic state remains unknown. By using isolated symbiotic endodermal cells, we focus to investigate how illumination used to provide photosynthesis and culture isolated zooxanthellae can affect zooxanthellae *in hospite* (*i.e.* zooxanthellae that are included in endodermal cells). A novel microscopic application where digital fluorescence microscopy is combined with *P*ulse *A*mplitude *M*odulated microscopy (Microscopy-PAM), was used to examine photosynthetic efficiency (Fv/Fm) of zooxanthellae. Upon illumination (0-100 $\mu E m^{-2} s^{-1}$), photosynthesis efficiency of cultured isolated zooxanthellae remains un-altered, while those of *in hospite* zooxanthellae significantly decrease in a dose-dependent manner. Illumination initiates the breakdown and permeabilization of membrane in symbiotic endodermal cells but not isolated zooxanthellae, indicating the presence of a membrane structure in symbiotic endodermal cells that is sensitive to illumination. Finally, changes of protein expression in symbiotic endodermal cells were specifically analyzed. Results show that a protein with molecular weight ~36 kDa decreased upon illumination in a dose-dependent manner. The disappearance of 36 kDa protein upon illumination is not due to the membrane breakdown. These results suggest that both specific membrane and protein may be critical in regulating the endosymbiosis in cnidarian species.

2733

**Translocation of Symbiotic Zooxanthellae from Ectoderm to Endoderm during the Development of Planula Larvae in Coral *Euphyllia glabrescens***

H. Huang,[1] L. Fang,[2] C. Chen[3]; [1]Research and Planning, National Museum of Marine Biology & Aquarium, Checheng, Pingtung, Taiwan Republic of China, [2]Institute of Marine Biodiversity and Evolution, National Dong Hwa University, Checheng, Pingtung, Taiwan Republic of China, [3]Institution of Marine Biotechnology, National Dong Hwa University, Checheng, Pingtung, Taiwan Republic of China

In endosymbiotic Cnidaria-dinoflagellate associations, sites of initial zooxanthellae (*i.e. Symbiodinium* spp.) acquisition, distribution and their effects on host animal development are unclear. Taking advantage of various autofluorescence expressed by larval tissues, we followed the distribution of symbiotic zooxanthellae during the process of larval release, settlement and metamorphorsis in a stony coral *Euphyllia glabrescens.* Results showed that planula larvae freshly released from parental animals already contain differentiated oral aperture in the region of blastopore, indicating the complement of gastrulation and formation of two distinct germ layers, the ectoderm and the endoderm. At this early stage, 70-80% of symbiotic zooxanthellae distribute in the ectoderm layer. During the following developmental process, symbiotic zooxanthellae translocate from the ectoderm into the endoderm by passing through the extracellular matrix mesoglea. Later on, all zooxanthellae (~100%) re-distribute into endodermal cells during the early stage of metamorphosis when tentacles start to emerge around the oral region. Quantitative analysis indicates that symbiotic zooxanthella is able to translocate in a directional manner between different germ layers either passively or actively during early development. Secondly, cellular sites of initial zooxanthellae acquisition and final establishment of stable endosymbiosis are different. Finally, the dynamic re-distribution of intracellular zooxanthellae between different germ layers may act as a pivotal signaling in determining the embryonic development of corals.

2734

**Searching the Site of Interaction between Microorganisms and Antimicrobial Peptides from the Hemocytes of the Spider *Acanthoscurria gomesiana***

A. H. Fukuzawa,[1] B. C. Vellutini,[2] P. I. Silva Júnior,[3] R. A. Mortara,[4] J. M. C. da Silva,[2] S. Daffre[1]; [1]Parasitologia, Universidade de São Paulo, São Paulo, Brazil, [2]Histologia e Embriologia, Universidade de São Paulo, São Paulo, Brazil, [3]Laboratório de Artrópodes, Instituto Butantan, São Paulo, Brazil, [4]Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo, Brazil

Invertebrates protect themselves against microbial infection by production of different antimicrobial peptides (AMPs). We previously presented the purification and characterization of two AMPs, gomesin and acanthoscurrin, from the hemocytes of naïve spider *A. gomesiana* (*J. Biol. Chem.*, 275: 33464, 2000; *Develop. Comp. Immunol.*, 27: 781, 2003). We also showed that gomesin is stored in granules of the hemocytes (*Insect. Biochem. Molec.*, 33: 1011, 2003). The main aim of the current work was to investigate the site of interaction between those AMPs and invader microorganisms: (i) in the plasma, after releasing of AMPs by the hemocytes or (ii) in the phagossomes after the fusion of granules containing AMPs. Firstly, the localization of gomesin and acanthoscurrin was compared by immunofluorescence, showing that 57% of hemocytes store both AMPs, either in the same granule or in different granules. Those AMPs were detected in the plasma after an experimental infection of spiders. Interestingly, despite phagocytosis be a conserved immune response described for almost all groups of arthropods, we did not observe the occurrence of such process after microbial challenge with *Sacharomyces cereviseae* in the spiders heart. In addition, it was noticed that the heart of spider presents some cells without granules, which probably are prehemocytes. Moreover, a challenged heart exhibited more hemocytes with granules than the naïve one, indicating a possible migration of these cells to the site of injury. Therefore, our results strongly suggest that the hemocytes might first migrate to the site of injury and then release their AMPs. Phagocytosis should have a subsequent role, being responsible for the clearance of cellular debris and remodeling of damaged tissues. Supported by FAPESP/CNPq

## Signal Transduction in Development (2735-2760)

2735

**Inositol Polyphosphate Pathway Regulates Vertebrate Left-Right Morphogenesis**

B. Sarmah,[1] B. Appel,[2] S. R. Wente[1]; [1]Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, [2]Biological Sciences, Vanderbilt University, Nashville, TN

Vertebrate body plans have a conserved left-right (LR) asymmetry manifested in the position and anatomy of the heart, visceral organs, and brain. Recently, we reported that the inositol 1,3,4,5,6-pentakisphosphate ($IP_5$) 2-kinase (Ipk1), an enzyme in the inositol polyphosphate (IP) pathway, is critical for the establishment of LR asymmetry in zebrafish (Sarmah et al., Dev. Cell 9, 133-145, 2005). Ipk1 catalyzes the conversion of $IP_5$ to inositol hexakisphosphate ($IP_6$). *ipk1* knockdown by antisense morpholino oligonucleotide injection randomizes LR-specific gene expression and organ placement, effects that are associated with reduced left-biased intracellular $Ca^{2+}$ flux in cells surrounding the ciliated Kupffer's vesicle (KV). A role for asymmetric $Ca^{2+}$ flux, potentially generated by cilia driven nodal flow, in establishing LR asymmetry in mice has also been reported by others. However, current understanding of the mechanisms that trigger and propagate this $Ca^{2+}$ flux in any vertebrate is poor. Strikingly, we observed that a reduction in Ipk1 activity is also associated with a decrease in KV-cilia beating raising the possibility that Ipk1 might mediate ciliary function. Zebrafish embryos express *ipk1* symmetrically in the axial mesendoderm and KV region. Ipk1 localizes uniformly throughout the cell. *ipk1* knockdown lowers production of $IP_6$ and more highly phosphorylated IPs (e.g., inositol pyrophosphate $IP_7$), and results in accumulation of precursor IPs (e.g., inositol tetrakisphosphate $IP_4$ and $IP_5$). Interestingly, knockdown of *ipk2*, encoding an inositol polyphosphate kinase for $IP_4$ and $IP_5$ synthesis, results in a phenotype comparable to *ipk1* knockdown. In contrast, embryos deficient in the $IP_6$-kinase exhibit an axis defect comparable to embryos overexpressing *ipk1*. Overall, our studies for the first time link the IP pathway, $Ca^{2+}$ signaling, and ciliary function together in mediating early events in LR morphogenesis and set a precedent for discovering future roles of IPs in vertebrate development.

2736

**PINCH Regulates Epithelial Morphogenesis in *Drosophila* Embryonic Dorsal Closure Where it Stabilizes Integrin Complexes**

M. A. Smith,[1] M. C. Elias,[2] J. L. Kadrmas,[2] S. M. Pronovost,[3] M. C. Beckerle,[1,2] R. A. Montague,[4] D. P. Kiehart[4]; [1]Department of Biology, University of Utah, Salt Lake City, UT, [2]Department of Oncological Sciences, University of Utah, Salt Lake City, UT, [3]University of Utah, Salt

Lake City, UT, [4]Developmental, Cell and Molecular Biology Group, Department of Biology, Duke University, Durham, NC

The coordination of integrin-mediated adhesion with signaling cascades is critical for developmental processes such as cell adhesion and motility. During *Drosophila melanogaster* embryonic dorsal closure, both integrin-mediated adhesion and signaling and Jun N-terminal kinase (JNK) signaling are required for proper migration and suturing of embryonic epithelia. We have previously shown that *Drosophila* PINCH, a five LIM domain scaffolding protein required for integrin related functions in the wing and for actin-membrane anchorage in muscle, is required for dorsal closure and for regulation of JNK signaling. In *Drosophila* embryos PINCH co-localizes with β-integrin, where it is concentrated at phosphotyrosine-rich adherens junctions in the leading edge cells of the migrating lateral epithelia. PINCH also colocalizes with β-integrin at cell-cell junctions in the amnioserosa. We have performed an analysis of embryos lacking maternally contributed PINCH. We show B-integrin stability and localization to the leading edge and amnioserosa is dependent on PINCH, and β-integrin levels are decreased in whole embryos lacking PINCH. However, removal of zygotic PINCH from β-integrin mutants does not exacerbate the *myospheroid* cuticle defect. PINCH is also essential for normal distribution of phosphotyrosine activation at the epithelial leading edge. Additionally, live analysis of PINCH mutants shows defects in stability of cell adhesion, and disruption of tissue migration, including proper assembly and function of the actin purse string at the epithelial leading edge. Our findings reveal multiple roles for PINCH in normal integrin function in *Drosophila* embryonic dorsal closure and suggest a general role for PINCH in signal integration during epithelial morphogenesis.

2737

### Initial Studies of FAK in *Ascidia ceratodes* Sperm Activation

E. Soria, R. A. Koch; Biology, California State University, Fullerton, Fullerton, CA

In ascidians, sperm activation also known as mitochondrial translocation (MTL), is characterized by the sperm mitochondrion binding to the outer surface of the egg complex and translocating off the head and down the tail. MTL is initiated by sperm-egg contact and involves cell-signaling communication that triggers actin cytoskeletal reorganization and myosin activation. We proposed that FAK, a key mediator of integrin-initiated signaling, responds to integrin-dependent sperm-egg adhesion and anchoring of the sperm mitochondrion to follicle cells located on the outer surface of the egg complex by signaling to cytoskeletal remodeling as required for MTL and sperm penetration. Previous work, has shown that integrins are present in *Ascidia ceratodes* sperm and required in MTL and fertilization. Anti-FAK and specific phospho-tyrosine antibodies, were used in indirect immunofluorescence to confirm the presence of FAK proteins in cells. Based on analysis of the genome database for the ascidian *Ciona intestinalis* we found that Ciona FAK-like proteins have several pentapeptides with a central tyrosine (Y) residue that align to human FAK sequences at the following sites, ciFAK Y260 to hsFAK Y397, ciFAK Y268 to hsFAK Y407, ciFAK Y440 to hsFAK Y576 and ciFAK Y441 to hs FAK Y577 and ciFAK Y671 to hsFAK Y861. Our data indicated that hs FAK Y925 did not have a Ciona counter part. The similarity of peptide sequences that contain tyrosines known to be phosphorylated during adhesion-induced signaling is being exploited to identify tyrosine residues that are involved in ascidian sperm activation. Our data support the presence of FAK in ascidian sperm and predict a role for FAK in adhesion-initiated sperm activation and ultimately in fertilization. (Funded by NIHR25GM56820).

2738

### The Hormonal Herbicide, 2,4-D, Induces a Signaling Response in Xenopus Oocytes that Interferes with Meiotic Maturation In Vitro

B. Stebbins-Boaz,[1] A. Compton,[1] A. LaChapelle,[2] M. Monnie,[1] J. Oost,[1] M. Ruygrok,[1] J. Swenson[1]; [1]Biology, Willamette University, Salem, OR, [2]Biology, LaChapelle, Salem, OR

Oocytes must undergo meiotic maturation for fertilization and embryo formation. In oocytes from the frog, Xenopus, this is triggered in vitro by progesterone, which activates the Mos-MAPK-p90rsk-cdc2/cyclin B1 signaling cascade leading to germinal vesicle breakdown (GVBD). Environmental contaminants that mimic hormones, endocrine disruptors, could potentially interfere with this pathway thus leading to poor egg formation and lowered fertility. Global amphibian declines may be explained in part by such effects. We have examined the oocyte response to the commonly used hormonal herbicide, 2,4-D, and found that it blocks maturation in vitro. Previous data showed that 2,4-D triggered MAPK phosphorylation while blocking Mos expression, cdc2/cyclin B1 activation and GVBD. To further characterize 2,4-D's mode of inhibition, we hypothesized that 2,4-D-activated MAPK does not fully activate the known maturation target, p90rsk. Western analysis showed partial phosphorylation of p90rsk in 2,4-D-treated oocytes relative to progesterone controls. To determine whether the failure of Mos expression in the presence of 2,4-D is due to decreased Mos stability, mature oocytes were incubated in 2,4-D for various times and subjected to western analysis. Results showed that Mos was stable once synthesized. Thus, 2,4-D does not by itself induce Mos degradation. We are investigating whether 2,4-D blocks Mos expression at the translational level by inhibiting Mos mRNA polyadenylation. Finally, to investigate whether 2,4-D acts upstream of Mos, we have examined whether 2,4-D recruits the endogenous maturation inhibitor, protein kinase A (PKA). Oocytes were microinjected with the PKA-inhibitor, PKI, and treated with 2,4-D. Controls showed that PKI, alone, induced maturation as expected. Preliminary results showed that 2,4-D inhibited this response suggesting that PKA is not an essential factor in 2,4-D-induced inhibition and that 2,4-D targets one or more downstream factors.

2739

### Involvement of Tyrosine Kinases, Specifically Src Family Kinases, Focal Adhesion Kinase (Fak), and Agonist-Induced PLC in the Activation and Development of Bovine Oocytes

B. J. Pate,[1] K. L. White,[1] D. B. DeWald,[2] L. F. Rickords,[1] G. Li,[1] K. I. Aston,[1] B. R. Sessions,[1] T. D. Bunch[1]; [1]Animal, Dairy, and Veterinary Sciences, Utah State University, Logan, UT, [2]Department of Biology, Utah State University, Logan, UT

In addition to contributing a genetic element to the oocyte at fertilization, the sperm cell induces the oocyte to leave its arrested state and resume metabolism in a process referred to as activation. A key to mammalian oocyte activation is release of intracellular calcium stores resulting in repetitive transients. The mediators of bovine activation are unknown at present, but are believed to be either a sperm-oocyte receptor interaction or a soluble cytosolic factor released by the sperm into the oocyte following fertilization. In either case, intracellular signaling pathways are initiated and perpetuated in such a way that the oocyte is induced to develop. The pathways leading to bovine oocyte activation have not been elucidated. Here we use known inhibitors of various intracellular signaling molecules to increase understanding of pathways involved in bovine oocyte activation. The tyrosine kinase inhibitor Genistein inhibited activation, while tyrosine kinase activator sodium orthovanadate induced activation.

The specific Src family kinase inhibitor PP1 also inhibited activation. In addition, an antibody directed against focal adhesion kinase, a mediator of integrin associated pathways, blocked oocyte activation. Wortmannin, a potent inhibitor of PI3-kinase had no effect on oocyte activation. These data indicate the involvement of tyrosine kinases, specifically one or more Src family kinases and focal adhesion kinase. We also used the phospholipase C inhibitor U73122 to block development and intracellular calcium transients. Inhibitor U73122 functions by blocking ligand-induced activation of PLC isoforms. An inactive analogue of U73122 referred to as U73343 had no significant effect on release of intracellular calcium. Together these data provides support for the receptor-mediated hypothesis of bovine oocyte activation by demonstrating the requirement of kinases and PLC isoforms associated with outside-in signaling.

2740

### *Drosophila* Mnk (Chk2) is Required for Inhibition of the Mid-blastula Transition in Response to DNA Damage

S. Takada, W. E. Theurkauf; Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

The mid-blastula transition (MBT) is the switch from maternal to zygotic control of early embryogenesis and is characterized by activation of zygotic gene transcription. It is preceded by a progressive increase in cell-cycle length and followed by dramatic morphological changes of the embryo. In *Drosophila melanogaster*, embryonic development initiates with rapid 13 syncytial divisions. Interphase (S phase) length in early cycles is only 5min and dramatically increases after division cycle10, up to 60-70 min for interphase14. Sudden increase of zygotic gene transcription is observed during interphase14, and cellularization and gastrulation take place. Embryos from DNA replication checkpoint mutant such as *grp* (DmChk1) and *mei-41* (DmATR) are defective in the pre-MBT cell-cycle lengthening. They also do not activate zygotic transcription and die without going through proper morphological changes (Sibon et al.1997 Nature 388:93; 1999 Curr. Biol. 9:302). Here, we show that a null mutation in Mnk (DmChk2) gene rescues the defective MBT in *grp* embryos without rescuing the pre-MBT cell cycle defects. *mnk grp* double-mutant embryos go though fast cell cycles like *grp* embryos, however, they activate zygotic transcription, cellularize and gastrulate. Injection of the DNA damaging reagents into wild-type embryos immediately before the MBT inhibits cellularization, however, mutant embryos lacking Mnk (DmChk2) cellularize. Chk2 is a conserved kinase that transduces signals leading apoptosis, DNA repair or cell-cycle checkpoint activation in response to DNA damage. We previously showed that Mnk is required for inhibiting chromosome segregation and also eliminating damaged nuclei from the embryo proper in response to DNA damage during the pre-MBT. Our results indicates that Mnk (DmChk2) transmits the DNA damage signal and inhibits the MBT and prevents further development of the embryo in the presence of DNA lesions.

2741

### Structural Basis for Notch Receptor Glycosylation by Fringe

M. Jinek,[1] S. M. Cohen,[2] E. Conti[1]; [1]Structural and Computational Biology Unit, European Molecular Biology Laboratory, Heidelberg, Germany, [2]Developmental Biology Unit, European Molecular Biology Laboratory, Heidelberg, Germany

The Notch pathway is an evolutionarily conserved pathway that controls many aspects of metazoan development. Modification of the Notch receptor by Fringe modulates the interactions between the receptor and its ligands, and constitutes a mechanism for establishment of tissue boundaries in *Drosophila* and vertebrates. Fringe is a Golgi-resident glycosyltransferase that transfers an N-acetylglucosamine (GlcNAc) monosaccharide from UDP-GlcNAc to specific fucosylated epidermal growth factor (EGF)-like repeats found in the extacellular domain of Notch and its ligands. We have determined the X-ray crystal structure of murine Manic Fringe at 1.8 Å resolution. Despite the lack of sequence similarity, the catalytic domain resembles those found in other glycosyltransferases, such as the *Bacillus subtilis* glycosyltransferase SpsA and rabbit N-acetylglucosaminyltransferase I. Based on these comparisons, we have identified residues responsible for substrate binding and catalysis and suggest a mechanism for the Fringe-mediated glycosylation of Notch.

2742

### Characterization of DetaD Endocytosis and Recycling

G. Palardy, M. Goswami, A. Chitnis; Laboratory of Molecular Genetics, NICHD NIH, Bethesda, MD

To understand the mechanism by which Delta internalization and ubiquitylation promotes activation of its receptor, Notch, we have investigated trafficking of fluorescent-tagged forms of deltaD in cell culture using Hela and Cos7 cell lines. The zdD2 monoclonal antibody, which recognizes the deltaD extracellular domain was used to monitor Notch-independent deltaD trafficking, while a soluble form of the Notch2 receptor (NECD-Fc), including the Notch2 extracellular domain (NECD) fused to an Fc fragment, was used to monitor Delta trafficking following interaction with its receptor. We find that pre-clustering of NECD-Fc is required for effective interaction with DeltaD and the interaction promotes Delta ubiquitylation in COS7 cells. Internalized deltaD co-localizes with proteins associated with both clathrin and non-clathin mediated endocytosis, suggesting it can be internalized by different mechanisms. In addition, DeltaD is resistant to TX-100 extraction following interaction with pre-clustered NECD-Fc, while it is efficiently stripped from the plasma membrane in the absence of NECD-Fc. This suggests that association with Notch segregates Delta to a distinct compartment of the plasma membrane that could bias its internalization mechanism and subsequent trafficking. DeltaD co-localizes with rab-11-GFP and with rab22-GFP, consistent with it eventually entering a recycling pathway associated with non-clathrin mediated endocytosis. The GTP-locked Arf6 mutant, Arf6(Q67L), which prevents passage of proteins internalized through a non-clathrin dependent endocytosis to the sorting endosome, reduces the amount of deltaD on the surface available to bind NECD-Fc. Rab22(Q64L), a mutant that interferes with recycling of non-clathrin dependent cargo also reduces the amount of deltaD on the plasma membrane. In contrast to effects of interfering with recycling, inhibition of protein synthesis for 2 hours does not significantly reduce surface DeltaD. Together these observations suggest DeltaD internalization and recycling may determine availability of Delta to the Notch receptor at the cell surface.

2743

### Enhancers and Suppressors of the Smoothened Tumor Suppressor

D. J. Casso, T. B. Kornberg; Biochemistry and Biophysics, UCSF, San Francisco, CA

The Hedgehog (Hh) protein acts as a critical regulator of development in a range of organisms from insects to vertebrates, and many of the downstream effectors of Hh signaling are corrupted in human cancers. Response to the Hh signal is regulated by two transmembrane proteins: Patched (Ptc) which binds Hh and negatively regulates signaling, and Smoothened (Smo) which is required downstream of Ptc to positively

regulate the pathway. While Smo is predicted to have a 7-transmembrane topology, it is not known how it controls Hh signaling. To identify novel components of the Hh pathway, we used a genetic screen to identify enhancers and suppressors of Smo in the fruit fly Drosophila melanogaster. We created a fly line in which Smo activity is reduced by expressing a Smo RNAi in the wing, causing a weak "fused" phenotype which is characteristic of a reduced response to Hh. We show that this phenotype can be suppressed by ectopic expression of Smo, or by heterozygous loss of function mutations in Ptc or Costal-2, both negative regulaors of Hh signalling. In addition, we show that heterozygous loss of function mutations in Hh, Smo, and Engrailed (all of which activate the pathway) enhance the phenotype. By screening a collection of chromosomal deletions on the second and third chromosomes, we identified 12 enhancer and 6 suppressor loci, including the loci of the above mentioned genes. The identification of the Smo enhancer and suppressor genes at these loci is underway.

2744

### *Drosophila* Hedgehog Signaling Effect on HRP-Smoothened Subcellular Localization Visualized using SBF-SEM

J. Buchanan,[1] A. J. Zhu,[2] R. Y. Arakaki,[1] S. J. Smith,[1] W. Denk,[3] M. P. Scott[2]; [1]Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA, [2]Developmental Biology, Stanford University School of Medicine, Stanford, CA, [3]Department of Biomedical Optics, Max-Planck Institute for Medical Research, Heidelberg, Germany

Hedgehog (Hh) is a secreted protein that influences patterning and morphogenesis in *Drosophila*. The regulation of Hh signaling by its receptor Patched (Ptc) and activator Smoothened (Smo) may involve intracellular trafficking through the late endosomal-lysosomal pathway. Following Hedgehog activation, the trans-membrane protein Smo accumulates at the cell surface in *Drosophila* salivary gland cells. In this study, we have used a functional HRP-Smo fusion protein to investigate Smo localization in response to Hh signaling by TEM (Transmission Electron Microscopy). In addition, we used SBF-SEM (Serial Block Face Scanning EM , Denk and Horstmann, *PLoS Biology* Vol. 2, No. 11, e329 DOI: 10.1371 ) to collect image stacks. This method allows collection of 3D data using automated serial sectioning by a special ultramicrotome mounted inside the SEM vacuum chamber. The block face is imaged after each section using backscattered electrons creating a TEM-like image. By examining image stacks of salivary glands from transgenic flies expressing *HRP-smo*, we were able to follow the distribution of the electron dense HRP-smo in 1000 thin sections (50μm) of tissue. The HRP-Smo was localized to numerous small clathrin coated vesicles, a small number of lysosomes and multivesicular bodies (MVBs). Following the addition of Hh protein, the HRP-Smo accumulated in dense patches between the digitated membranes of neighboring epithelial cells. Small HRP-Smo containing vesicles accumulated along the lateral cell membranes. Larger tubular structures containing HRP were also evident in this region. There was a large increase in HRP- positive lysosomes following Hh stimulation. The combination of HRP fusion protein and SBF-SEM greatly improves the precise localization and 3D distribution of Smoothened in Hh+ and Hh- treated salivary glands.

2745

### CKIε Regulates Wnt-Mediated Small G Protein Activation by Stimulating the Degradation of a GTPase Activation Protein

I. Tsai, Z. Gao, D. M. Virshup; Department of Oncological Science, University of Utah, Salt Lake City, UT

Wnt signaling is an important signaling pathway for cell proliferation and embryonic development. Recent studies indicate Wnt signaling can activate small G protein pathways including as RhoA and Rac, promoting cell planar polarity and cell motility. Casein kinase I epsilon (CKIε) is a positive regulator of the canonical Wnt signaling pathway, and previous studies have shown the activity of CKIε is stimulated by Wnt signaling. To understand if CKIε is also involved in Wnt-mediated small G protein activation, we screened for additional binding partners of CKIε by using the two hybrid method. Here we report identification of an upstream regulator (a GTPase activation protein (GAP) protein) of a Ras- family small G protein. CKIε interacts with this GAP protein and reduces its stability in mammalian cells. Similarly, overexpression of CKIε rescues the inhibition of the small GTPase by the GAP protein. Upon stimulation by Wnt-conditioned medium, the GTP-bound form of the Ras-family small G protein is increased. However, activation of this small G protein by Wnt signaling was blocked by the specific inhibitor of CKIε, IC261, implying CKIε is required for this Wnt-mediated small G protein activation. Treatment of IC261 also leads to the reduction of Wnt-mediated cell motility. Our findings support a model that Wnt signaling activates CKIε, promoting the degradation of a GAP protein of Ras-family small G protein, leading to the activation of this small G protein pathway and cell motility.

2746

### Characterization and Manipulation of G protein-mediated Signal Transduction in *Dictyostelium*

B. Raisley, W. Watson, S. Burch, J. Griffin, J. Melton, J. Hadwiger; Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK

The soil amoeba *Dictyostelium discoideum* detects and forages on bacteria, but when depleted of nutrients it undergoes multicellular development with spore production. The sensing of folic acid released from bacteria by *Dictyostelium* stimulates cyclic nucleotide accumulation, $Ca^{2+}$ influx, chemotaxis, and gene regulation through a Gα4-specific G protein-mediated pathway. The use of this pathway as the foundation for the development of a biosensor, studying the specificity of G protein function, and the identification of a folic acid receptor gene are areas of investigation. As a natural detector of folic acid, *Dictyostelium* is an excellent candidate for utilization as a biological sensor of bacteria. To use *Dictyostelium* in this manner, a GFP-apoaequorin gene fusion was created and expressed from different promoters. The bioluminescent output of the encoded proteins is expected to allow rapid detection of $Ca^{2+}$ influx in *Dictyostelium* stimulated by folic acid. The specificity of Gα subunit function in this pathway was tested by expressing chimeric Gα2 and Gα5 subunits with a Gα4-specific receptor interaction domain. These proteins, Gα2/4 and Gα5/4, were used in attempts to restore responses to folic acid in cells lacking the Gα4 subunit. The Gα2/4 subunit partially restored some responses, but the Gα5/4 did not and even inhibited responses in cells with a functional Gα4 subunit. These results suggest that Gα subunit specificity is not limited to receptor interaction domains. Because a folic acid receptor has yet to be characterized in *Dictyostelium*, a search is being conducted by disrupting genes that encode putative heptahelical proteins. These putative receptors share sequence identity with human $GABA_B$ receptors and might function as heterodimers. This receptor search and analysis is in collaboration with Dr. Dale Hereld at the University of Texas-Houston Medical School.

2747

**PldB, a Putative Phospholipase D Homologue Negatively Regulates Quorum Sensing in *Dictyostelium discoideum* Development**

Y. Chen, V. Rodrick, Y. Yan, D. Brazill; Biological Sciences, Hunter College, City University of New York, New York, NY

Quorum sensing, the ability to measure the local cell density, is required for animal cells to achieve proper cell growth, differentiation and development. However, because of the genetic intractability of mammalian cells, we study quorum sensing in the genetically pliable unicellular eukaryote *Dictyostelium discoideum*. *Dictyostelium discoideum* cells live as individual cells under vegetative condition. When starved, they are able to calculate the concentration of surrounding starving cells by simultaneously secreting and sensing a glycoprotein named conditioned medium factor (CMF). A high density of starving cells ($>10^5$cells/cm$^2$) results in a high level of CMF, which allows cell aggregation and development. Here, we describe the role of a putative phospholipase D (PLD) homologue, PldB, in quorum sensing pathways. PldB is present during early development when quorum sensing is occurring as well as in later development. *pldB*$^-$ cells can aggregate at low cell density, while cells overexpressing *pldB*are unable to aggregate or form fruiting bodies even at high cell density, implying that PldB is a negative regulator of quorum sensing. This phenotype is cell autonomous since mixing a small number of *pldB*$^-$ cells with wild-type cells does not make the wild-type cells behave like *pldB*$^-$ cells, nor does a small number of wild-type cells make *pldB*$^-$ cells behave like wild-type cells. *pldB*$^-$ cells develop faster than wild-type cells, indicating that *pldB* is involved in the timing of development. Analysis of early developmental gene expression shows that cAMP receptor cAR1, used in aggregation, is expressed at higher levels earlier in *pldB*$^-$ cells than in wild-type cells, which may explain the rapid aggregation phenotype of *pldB*$^-$ cells. Moreover, western blot analysis and GFP-tagged PldB reveal that PldB protein is localized to cytoplasmic membrane as well as vesicles.

2748

**Identification and Characterization of $GABA_B$ Receptor like-proteins in *Dictyostelium discoideum***

Y. V. Prabhu, A. A. Noegel; University of Cologne, Center for Biochemistry, Medical Faculty, Cologne, Germany

GPCRs are a superfamily of diverse integral membrane proteins that allow detection and transduction of a large variety of extracellular signals. In *Dictyostelium* only seven members of one family, the crl (cAMP receptor like) family, had been identified and studied in detail. The analysis of the genome sequence uncovered 48 additional putative GPCRs grouped into the secretin (family 2), metabotropic glutamate/GABA B (family 3) and the frizzled / smoothened (family 5) families of receptors. The presence of family 2, 3 and 5 receptors in *Dictyostelium* was indeed surprising because they had been thought to be animal-specific. GABA (gamma amino butyric acid), the principal inhibitory neurotransmitter in mammalian brain, signals through ionotropic ($GABA_{(A)}$/$GABA_{(C)}$ ) and metabotropic $GABA_{(B)}$ receptor systems. The functional $GABA_B$ receptor is a heterodimer of receptor 1 and receptor 2 subtypes. The *Dictyostelium* genome harbours 17 different genes encoding $GABA_B$ receptor like proteins of which 15 resemble the subtype 2 and two appear to be closer to the subtype1. Yeast two hybrid studies led us speculate that the C termini of these receptors alone may not be sufficient for their interaction(s) to form heterodimers. The detailed analysis of one of each type of receptors named as GrlA and GrlJ was undertaken. Both these receptors are expressed throughout the development of *Dictyostelium*. To gain more insight into the *in vivo* function of these proteins, we generated knockout mutants by homologous recombination. Mutant analysis revealed GrlA to be a player in the later developmental stages acting in a stalk specific manner by displaying delayed expression of a prestalk specific gene, ecmB.

2749

**Expression and Role of CaMK-II during Zebrafish Development**

R. M. Tombes,[1] C. R. Barrett,[1] J. A. Lister[2]; [1]Biology, Virginia Commonwealth University, Richmond, VA, [2]Human Genetics, Virginia Commonwealth University, Richmond, VA

CaMK-II is a $Ca^{2+}$/calmodulin-dependent protein kinase that is encoded by four genes (α, β, γ, and δ) and has widespread and pleiotropic roles throughout the lifespan of metazoan organisms. The role of CaMK-II in early vertebrate development was examined in the zebrafish, *Danio rerio*. All four CaMK-II genes have recently been identified in zebrafish and δ CaMK-II expression has been detected in early embryos. We have found that total CaMK-II activity is expressed rapidly upon fertilization, increasing by 20-fold between one and three days of development. Zebrafish embryos were injected soon after fertilization with a CaMK-II anti-sense morpholino oligonucleotide designed against the first exon of zebrafish δ CaMK-II. Injected embryos exhibited developmental aberrations including diminished, curved tails, jaw deformities, and heart irregularities. KN-93, a CaMK-II antagonist, suppresses the activity of all CaMK-II isozymes. Phenotypes of embryos incubated with KN-93 were similar to those seen with the morpholino oligonucleotide in that circulatory system defects as well as jaw and body axis length abnormalities were observed in a concentration-dependent manner. The proposed involvement of CaMK-II in the Wnt pathway is supported by the similarity of slb and ppt mutant phenotypes (associated with Wnt11 and Wnt5a respectively) to the CaMK-II morpholino phenotype. Our findings indicate that CaMK-II is a naturally expressed developmental kinase that is necessary for the proper development of the heart/circulatory system and may be required for the formation of a linear spine.

2750

**Expression Analyses of Profilins and their Effects on Zebrafish Development**

T. Chan, C. Wu, S. Lai, H. Chang, S. Lee; Institue of Zoology, National Taiwan University, Taipei, Taiwan Republic of China

Profilin, an actin-binding protein, has been demonstrated to be essential for cell division and survival in several animal species. The regulatory role of profilin in development is presumably via its mediation on actin microfilament assembly and profilin-associated signaling molecules. Massive reorganizations of actin microfilament are central to the proper progression in embryogenesis. However, little information is available regarding the characterization and functions of profilin in zebrafish embryos. By blasting zebrafish genome, we have identified a cDNA clone corresponding to a profilin II like protein, which we designate it as zpfnII here. Using RT-PCR analysis, a 423 bp zpfnII transcript is highly expressed in early embryonic stages and all adult tissues examined in zebrafish. However, by whole-mount in situ hybridization, zpfnII was found to be ubiquitously expressed in all blastomeres of early embryos, but was localized mainly in brain and neuronal tissues in later stage embryos. Overexpression of zpfnII-GFP caused defects in somite and neuron formation. Furthermore, morpholino knock-down of zpfnII induced a shorten body axis and more serious abnormality in body shape. These results suggest that zpfnII is required for proper neuron, somite and possibly body axis formation in zebrafish development. In addition, we also identified a splice variant of zpfnII with 782 bp (zpfnII782), which was strongly expressed in brain and

neuron in both adults and 1-2 days embryos in both RT-PCR and whole mount *in situ* hybridization analyses. It implies that zpfnII782 may play a more specific role in brain development and neurogenesis

2751

**Citron Kinase is Essential for Embryonic Development in Zebrafish**

M. Chien, S. Lee; Institue of Zoology, National Taiwan University, Taipei, Taiwan Republic of China

Citron kinase, a serine-threonine kinases, is a target molecule for activated Rho, which is involved in cytokinesis and embryonic development. While its role in cytokinesis is well documented, the effects of inhibition of citron kinase vary on species. The discribancy in citron kinases inhibition on the embryonic development may be due to the efficacy of gene inhibition, gene compensation or species differences. Zebrafish, a recent evolved vertebrate, whose gene functions can be effiiently knowcking down by morpolino antisense oligonucleotides (MO). To study the role of citron kinase in zbrafish development, we have isolated a 1.8 kb clone containing the N-terminal kinase doamin of zebrafish citron kinase by *in silica* cloning. We showed that citron kinase mRNAs were ubiquitously existed in different developmental stage embryos and adult zebrafish tissues by RT-PCR. By whole-mount in situ hybridization, we observed that citron kinase expressed in the entire embryo until 18 hour post fertilization and the expression of citron kinase mRNA was limited to the proliferative neuroblasts and tissues. Lastly, we demonstrated that knockdowning citron kinase by MO dose-dependently resulted in cytokinesis defects, irregular shapes of embryos, delayed growth and failure in epiboly, which sbsequently casued embryonic death within 24 h post fertilization. Altogether, these results suggest that citron kinase is essential for the early development in zebrafish.

2752

**Mesenchymal p66$^{Shc}$ Expression Induces Fetal Mouse Lung Branching Morphogenesis While Epithelial Expression Attenuates Branching**

M. K. Lee, S. M. Smith; Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA

Shc adaptor proteins mediate mitogenic signaling by a number of different cytosolic and membrane-bound kinases. In mammals, the Shc proteins are expressed as three isoforms with different and opposing functions. While the 52 and 46 kDa isoforms mediate activation of the Erk1/Erk2 MAP kinases, the 66 kDa Shc isoform (p66$^{Shc}$) sequesters Grb2 and antagonizes Erk activation. Unlike the ubiquitous 52 kDa isoform, p66$^{Shc}$ is developmentally regulated. p66$^{Shc}$ is highly expressed in both epithelial and mesenchymal tissues within the early fetal mouse lung, but expression decreases and becomes progressively restricted to epithelial cells as the lung matures. We hypothesized that lung branching morphogenesis is dependent on regulated p66$^{Shc}$ expression within these two tissues. To address this postulate, either an siRNA specific for p66$^{Shc}$ or an adenoviral vector encoding p66$^{Shc}$ was applied to the luminal or mesenchymal surfaces of fetal mouse lung explants at 12 days post-conceptual age. The explants were then cultured in serum-free defined medium for four days. To confirm cellular uptake, siRNA preparations were labeled with a non-silencing siRNA conjugated to Cy5, and the adenoviral vectors concomitantly expressed green fluorescent protein. Vector uptake was confirmed by epifluorescence microscopy, Shc isoform expression was evaluated by Western analysis, and lung branching was assessed morphologically. Induction of mesenchymal p66$^{Shc}$ was associated with increased airway branching while attenuated mesenchymal p66$^{Shc}$ expression correlated with reduced branching. In contrast, induced epithelial p66$^{Shc}$ expression coincided with reduced airway branching morphogenesis while attenuated epithelial p66$^{Shc}$ was associated with increased branching. These results suggest that p66$^{Shc}$ modulates lung branching morphogenesis, and that p66$^{Shc}$ signaling in mesenchymal cells has opposite effects on branching morphogenesis to epithelial p66$^{Shc}$ signaling.

2753

**Activation of FAK is Necessary for Osteogenic Differentiation of Human Mesenchymal Stem Cells on Laminin-5**

R. F. Klees,[1] R. M. Salasznyk,[1] A. Boskey,[2] G. E. Plopper[1]; [1]Biology, Rensselaer Polytechnic Polytechnic, Troy, NY, [2]Biology, Hospital for Special Surgery, New York City, NY

Mesenchymal stem cell differentiation into osteoblasts and the signaling events that shape this differentiation are poorly understood. We recently established that contact with specific extracellular matrix proteins, in particular laminin-5, is sufficient to induce an osteogenic phenotype in human mesenchymal stem cells (hMSC) through an extracellular signal-related kinase (ERK)-dependent pathway. Activation of ERK 1/2 by laminin-5 induces phosphorylation of the runx2/cbfa-1 transcription factor that controls osteogenic gene expression. We hypothesized that focal adhesion kinase (FAK) mediated signaling pathways supply a link between cell surface integrin-ECM binding and activation of ERK 1/2, and that laminin-5, a well known activator of FAK, promotes its osteogenic effects through this pathway. To test this hypothesis, we plated hMSC on a laminin-5 matrix in the presence or absence of FAK-specific small inhibitory RNAs (siRNA), and assayed for phosphorylation of runx2/cbfa-1 as well as expression of established osteogenic differentiation markers (bone sialoprotein 2, osteocalcin, alkaline phosphatase, calcium deposition, and mineral:matrix ratio). We found that siRNA treatment reduced total endogenous FAK protein by ~40%, and reduced FAK phosphorylation on Y397 in cells plated on laminin-5 for 30 minutes. hMSC transfected with FAK siRNA and plated on laminin-5 also exhibited a decrease in ERK 1/2 phosphorylation after 1 hr, and reduced serine phosphorylation of Runx2/Cbfa-1 after 8 days. Finally, FAK inhibition blocked osteogenic differentiation of hMSC, as assessed by lowered expression of osteogenic genes (RT-PCR), decreased alkaline phosphatase activity, greatly reduced calcium deposition, and a lower mineral:matrix ratio after 28 days in culture. These results establish FAK as an important mediator of laminin-5 induced osteogenic differentiation of hMSC.

2754

**Roles for the Tumor Suppressor Genes *Adenomatous Polyposis Coli 1* and *2* in Cell Adhesion and Axon Outgrowth in the Developing *Drosophila* Larval Brain**

M. A. Hayden,[1] K. Akong, [1] M. Peifer[2]; [1]Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Adenomatous Polyposis Coli (APC) is a multi-domain protein that was first characterized as a tumor suppressor, which acts as a negative regulator of the Wnt/Wingless (Wg) signaling pathway. Mutations in human *APC* lead to the development of colon carcinoma. APC acts in a complex with several other proteins to negatively regulate the levels of free β-catenin /Armadillo by helping to facilitate its phosphorylation, allowing subsequent ubiquitin-mediated destruction. In addition, APC has Wg-independent roles in the regulation of the cytoskeleton during normal development. Two

APC genes have been identified in both mammals and in *Drosophila* (*APC1* and *APC2*). We have found that both APC1 and APC2 play redundant roles in larval brain development. Using mosaic analyses, we generated GFP-marked *APC* double mutant clones in the larval brain to examine what roles APCs play in Wg-dependent and Wg-independent events. A null allele of APC1 combined with one of several alleles of APC2 result in varying degrees of structural aberrations in the larval brain. These defects arise in both epithelial tissues and axon projections. Double mutant cells segregate from their neighbors to form epithelial "loops," while double mutant neurons form tangled axonal "knots". We are currently examining how the defects seen relate to changes in cell adhesion, Wg signaling, and/or altered regulation of the cytoskeleton.

2755

**Structure-Function Analysis of the Neurogenic Gene Product Big Brain**

M. K. Renihan,[1,2] G. Boulianne[1,2]; [1]Developmental Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]University of Toronto, Toronto, ON, Canada

The neurogenic gene product Big brain, Bib, is essential for the development of the embryonic nervous system. *bib* mutant embryos display neural hyperplasia at the expense of ectodermal tissue. Although some evidence suggests that *bib* acts in the Notch pathway in a cell autonomous manner, little is known about *bib* function *in vivo*. *bib* is predicted to encode a protein containing a short N-terminal cytoplasmic region, 6 transmembrane domains and a long C-terminal cytoplasmic tail containing several potential regulatory regions and protein-protein interaction domains. Recently, studies using *Xenopus* oocytes as a model system have shown that Bib can function as a non-selective monovalent cation channel, and have revealed regions of this protein that affect its channel function and that undergo post-translational modification. However, whether channel function and post-translational modification are critical for Bib's role in *Drosophila* development or Notch signalling remains unclear. To determine which regions of Bib are necessary and/or sufficient for its function during *Drosophila* development and in Notch signalling we are employing the GAL4-UAS system and cell culture to express: (1) a deletion construct lacking the C-terminal tail of Bib; (2) various mutations in the first transmembrane domain that have been shown in *Xenopus* oocytes to affect Bib function; (3) various mutations in the C-terminal cytoplasmic tail and most C-terminal transmembrane domain; and (4) the entire Bib protein. Using a structure-function approach will provide insight into the role of Bib in Notch signalling and *Drosophila* development.

2756

**aPKCζ Signaling Regulates the Stratification of Neural Progenitor Cells**

S. Ghosh,[1] T. Marquardt,[2] J. P. Thaler,[2] S. L. Pfaff,[2] T. Hunter[1]; [1]MCBL, Salk Institute for Biological Studies, La Jolla, CA, [2]GEL, Salk Institute for Biological Studies, La Jolla, CA

The role of neuroepithelial cell polarity in controlling segregation of cell-fate determinants during stem cell divisions and in the developmental patterning of the central nervous system is of crucial importance, yet is poorly understood. Here we show that atypical Protein Kinase C ζ/λ (aPKCζ/λ) localizes at the apical 'end-feet' of radial progenitor cells in the developing chick spinal cord. Consistent with a role in regulating neuroepithelial polarity, mislocalized aPKCζ activity disrupts the polarized distribution of cell adhesion proteins and induces the premature delamination of undifferentiated neural progenitor cells, but the loss of cell polarity is dispensable for cell-fate specification during neurogenesis. aPKCζ integrates signals from the PI 3-Kinase and the Rac/Cdc42 pathways and the localized activation of aPKCz regulates the timing of neuronal precursor cell delamination and migration.

2757

**Role of Trio GEF in the Migration and Proper Organisation of Inferior Olivary Neurons**

S. Backer,[1] S. Marcos,[1] M. Hidalgo-Sanchez,[1] E. Portales,[2] P. Fort,[2] A. Debant,[2] C. Gauthier-Rouvière,[2] E. Bloch-Gallego[1]; [1]Institut Cochin - Département Génétique et Développement, INSERM U. 567, Paris, France, [2]CRBM-CNRS FRE 2593, Montpellier, France

During embryonic development, inferior olivary neurons (ION) first follow a circumferential migration from their birthplace in the alar part of the brainstem to the vicinity of the floor-plate, where they get packed in a club-shaped domain. Then ION organize into a laminated structure. As we had previously shown that the Rho GTPases Rac1/Cdc42 are involved in axon outgrowth of ION during tangential migration, whereas the Rho/ROCK pathway is involved in nucleokinesis, we aimed at further characterizing the regulators of Rho-GTPases activity that could direct the migration and organization of ION. We have analyzed the involvement of the guanine nucleotide exchange factor TRIO that contains two GEF domains for RhoG/Rac1 and RhoA. We have characterized the expression and activation of Rac, Cdc42 and Rho during ION development. We have studied the successive steps of ION development in TRIO deficient mice. In the absence of Trio, initial axon outgrowth and nucleokinesis occur properly but the second step of ION development to get a proper laminated structure is affected. Thus, other GEFs, involved in the control of the activities of Rac/Cdc42 and RhoA during the initial circumferential migration remain to be characterized and we further investigate potential upstream regulators of Trio involved in the maturation and organization of ION, with a special care on receptors of chemotropic factors and adhesion molecules.

2758

**A Role for Duplin in SRF-mediated Signaling**

J. M. Rodenberg, B. P. Herring; Cellular & Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN

Differentiated smooth muscle cells have the remarkable ability to switch between a quiescent contractile phenotype and a proliferative, synthetic phenotype under many pathological conditions. Serum response factor (SRF) is a transcription factor that is crucial for regulating genes characteristic of contractile smooth muscle cells as well as activating genes required for cell proliferation. The ability of SRF to regulate these two disparate phenotypes is dependent on its ability to interact with proteins that alter its activity towards differentiation genes as opposed to proliferation genes. Identifying and unraveling the function of these SRF-associated proteins is thus crucial for understanding the mechanisms regulating the phenotype of smooth muscle cells. A yeast two-hybrid screen using SRF as bait identified the protein Duplin. Duplin is a nuclear protein that was previously shown to bind to β-catenin and inhibit its signaling. Interestingly, knocking out Duplin is embryonic lethal, due to defects in gastrulation, although expression of β-catenin target genes was unaffected (Nishiyama, et al. 2004). SRF-GST fusion proteins demonstrate a direct interaction of SRF with the amino-terminal portion of Duplin. Preliminary results from mammalian two-hybrid experiments in

cos cells also indicate a direct interaction between SRF and Duplin. The phenotype of SRF knockout mice is similar to the Duplin knockout mice with a failure of mesoderm formation during gastrulation. Together these data suggest that Duplin may play an important role in modulating SRF-dependent gene expression. shRNA is currently being used to knock-down Duplin expression in smooth muscle and nonmuscle cells to determine the effects on SRF-dependent genes involved in differentiation and growth. Although much of the physiological significance of Duplin remains unknown, from our preliminary data we can conclude that Duplin binds SRF independently of possible interactions with β-catenin. Funding: NIH R01 DK61130 (BPH).

2759

**Smad7 Enhances Myogenesis by Inhibition of Myostatin but not Transforming Growth Factor-β Signaling**

H. D. Kollias, R. L. S. Perry, J. C. McDermott; Biology, York University, Toronto, ON, Canada

TGF-β and myostatin signaling mediated by the Smad downstream effectors, potently repress skeletal muscle cell differentiation. Based on the possibility that inhibition of these pathways might enhance myogenesis, the role of the inhibitory Smad (Smad7) during skeletal muscle cell differentiation was assessed. In these studies we document that increased expression of Smad7 differentially abrogates myostatin but not TGF-$\beta_1$ mediated repression of myogenesis. Further, constitutive expression of exogenous Smad7 enhanced skeletal muscle differentiation and cellular hypertrophy. Interestingly, targeting of endogenous Smad7 by siRNA inhibited C2C12 muscle cell differentiation indicating requirement for Smad7 during myogenesis. Congruent with a role for Smad7 in myogenesis we also observed that the Smad7 promoter is up-regulated by muscle regulatory factors, MyoD and myf5. Finally, we document that Smad7 can augment the myogenic conversion of 10T/2 fibroblasts by myf5. Taken together these data implicate Smad7 as a fundamental regulator of myostatin signaling and differentiation in skeletal muscle cells.

2760

**Possible Role for A Kinase Anchoring in Muscle Cell Differentiation**

A. Andrews, J. Camaren, J. George, A. Medford, S. K. Byrd; Biology, Fort Lewis College, Durango, CO

The objective of our study was to examine the involvement of A kinase anchoring proteins (AKAPs) in muscle cell differentiation. L6 myoblasts were initially grown in high serum, and then switched to low serum to promote differentiation. Cells were grown for a period of nine days in differentiation medium containing either 10 µM Ht31, a peptide that inhibits binding of the regulatory subunit of PKA (RII); 10 µM Ht31P, which does not block RII binding; or normal medium. Cells were harvested at 0, 3, 6, and 9 days of growth in differentiation medium. Fresh media and peptides were added daily. Western blots of whole cell lysates were examined for the production of skeletal specific myosin and for changes in AKAPs. We specifically examined changes in mAKAP, a 222Kd protein known to be present in the perinuclear area as well at near the ryanodine receptor in mature muscle. We found that the myoblasts incubated with Ht31 produced greater amounts of myosin sooner in the differentiation process. There was a 28% increase in myosin by day 6 and 90% by day 9, over the controls. This pattern of more rapid differentiation in the presence of Ht31 was mirrored by production of mAKAP, with progressively greater mAKAP at day 6 and at day 9. We suggest that up regulation of certain AKAPs and the associated changes in signaling scaffolds may play a role in muscle cell differentiation.

## Stem Cells II (2761-2779)

2761

**Microvascular Tubes Derived from Mouse Embryonic Stem Cells Sustain Blood Flow**

X. Zhou, I. Gallicano; Cell Biology, Georgetown University Medical Center, Washington, DC

The recent production of somatic cell nuclear transfer (SCNT)-derived embryonic stem cells has brought therapeutic cloning closer to reality. Among the potential applications of therapeutic cloning is therapeutic angiogenesis. While recent progress has been made with clinical therapeutic angiogenesis, it has been met with limited success. One reason for this limit has been the cell types used to generate the collateral vessels used for shunting around coronary blockages. Consequently, we developed a procedure using the embryonic stem (ES) cell model system to generate microvascular tubes similar to small vessels found in vivo. We then evaluated their ability to graft and sustain blood flow by transplanting them onto EGFP expressing E9 day embryo hearts. Microvascular tubes generated from embryonic stem cells have not been thoroughly tested for their ability to graft and function within the heart primarily because of issues including immune rejection of the foreign cells comprising collateral vessels, and limited methodologies to prevent teratoma risk. However, since recent therapeutic cloning techniques have provided evidence of diminished risk of immune rejection, we improved the methodology for generating and isolating tubes from ES cells to evaluate their applicability for therapeutic angiogenesis. Here, we demonstrate that microvascular tubes generated from ES cells are capable of grafting onto E9 day embryo hearts and sustaining the flow of blood cells as verified by EGFP expressing blood cells within non-EGFP ES cell-derived microvascular tubes.

2762

**Impact of Oxygen Tension on Proliferation and Differentiation of Hematopoietic Stem/Progenitor Cells (HSPC)**

T. McKinnon, N. Baal, K. Reisinger, M. T. Zygmunt; Obstetrics and Gynecology, University of Giessen, Giessen, Germany

Early human development commences under the influence of low oxygen concentration until vasculogenesis takes place. However, chronically low oxygen tensions are associated with early pregnancy losses due to vascular malformation. We describe *in vitro* culture system to mimic low oxygen environments during pregnancy [i.e. hypoxia (1% $O_2$), normoxia (8% $O_2$) and as a control, hyperoxia (21% $O_2$)] in order to investigate placental vasculogenesis using HSPC. Concurrent real-time oxygen concentrations were measured with Sensor Dish Reader 2 (PreSens, Regensburg, Germany). HSPC, which can contribute to the first vessel system, contain both endothelial and hematopoietic progenitor cells. CD133+ cells isolated from umbilical cord blood collected from term pregnancies have been extensively characterized as HSPC and used in models for vasculogenesis. We expand HSPC in media containing Flt3-ligand, stem cell factor and thrombopoietin; they express several pluripotency markers, including Oct4, Sox-1, Sox-2, FGF4 and Rex-1 at RNA and protein levels. HSPC cultured in hyperoxia proliferate rapidly for approximately 20 days and continue in culture for >40 days. Conversely, HSPC number in low oxygen tensions is maintained for >40 days without significant increases in cell number. Decreased proliferation of HSPC in low oxygen environments was not due to significant changes in the proportion of cells in G1/G0, S or G2/M. HSPC expansion for 7-10 days resulted in decreased CD133 and CD34 expression while further HSPC culture in low

oxygen resulted in significant increases in differentiation markers (CD14, CD11b) and still retain their potential to differentiate into endothelial cells. We have established a novel, low oxygen *in vitro* culture system. Using this system we demonstrated that HSPC grown in physiologic placental oxygen tensions switch from a proliferating to a more differentiated phenotype and display possible prerequisites for vessel assembly.

2763

**Hematopoietic Stem Cell Localization on a 3-D Matrix**

S. L. Snabes,[1] L. Noll,[1] D. Smith,[1] N. Kotov,[2] E. Elliott,[2] J. Bahng,[2] J. Lee,[2] L. Lasky[3]; [1]Research, Aastrom Biosciences, Ann Arbor, MI, [2]Chemical Engineering, Biomedical Engineering, Materials Science, University of Michigan, Ann Arbor, MI, [3]Pathology and Internal Medicine, University of Ohio, Columbus, OH

Modeling intricate microenvironments such as bone marrow has until recently proven difficult. Previous methods involved growing cells on 2-D surfaces, where cultures are frequently passaged or diluted and where differentiation pressures vary greatly from their natural state. These cultures result in an unnatural cellular organization that can only be maintained for limited periods of time. We therefore constructed a culture system that incorporates a 3-D substrate into a circumfusion flowloop where the local microenvironment differs based upon proximity to nutrients, gasses, and by-product concentration. We then compared this 3-D matrix/perfusion system to 3-D matrices in rotary bioreactors, spinner flasks, and static wells to ascertain the effects of gravity and perfusion on stem cell localization. Functional tissue and cellular development were ascertained by frequent flow cytometry, cytospins, colony assays, gene microarrays, lactate analysis, cell and viability counts, SEM and confocal microscopy. Our findings suggest that stem cell organization throughout a 3-D matrix differs greatly depending on how the matrix is integrated with its fluid and gaseous environment. Imaged cultures from the perfusion systems demonstrate tissue density with 80+% viabilities for up to 360+ days. Unlike static cultures where cells localize in layers on the matrix surface, the perfusion matrix revealed large aggregates of cells of varying morphology, function, and size in close association that form tissue density layers throughout the matrix depth. These cultures appear indicative of normal bone marrow (1.5%+ CD34+), allowing us to investigate the role of hypoxia and nutrient proximity on cellular localization. Furthermore, by utilizing an environment that allows for cells to release from marrow (matrix) to circulation (flow loop); we can analyze both cultured marrow tissue and circulating HSC's. Support: DAMD17-02-1-07-04

2764

**Deficiency in Gata-4 or Gata-6 Diminishes Definitive Hematopoiesis in Murine Embryonic Stem Cell Derived Embryoid Bodies**

M. S. Pierre, M. Yoder; Herman B Wells Center for Pediatric Research, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN

Previous investigators have reported that differentiation of embryonic stem (ES) cells deficient in either GATA-4 or GATA-6 results in embryoid body (EB) with disrupted differentiation of visceral endoderm (VE) and defective blood island formation. **OBJECTIVES**: In the current study, the ability of GATA-4 and GATA-6 null ES cell derived EB to commit to early hematopoietic lineages was compared to that of wild-type and heterozygous EB cells using secondary hematopoietic progenitor plating assays. **METHODS**: Cells dissociated from day 2 or day 3 EB plated into blast colony forming cell (BL-CFC) assays revealed no difference in wild-type, heterozygous or homozygous deficient EB ability to differentiate into hemangioblast. **RESULTS:** Analysis of primitive erythroid progenitors using cells dissociated from day 4, 5, 6 or 7 EB showed similar numbers of primitive erythroid progenitor colonies obtained from wild-type, heterozygous and homozygous deficient EB. Definitive hematopoiesis of EB was assessed by secondary plating of day 8, 9 and 10 EB into definitive erythroid and granulocyte/macrophage progenitor assays. The number of definitive erythroid progenitor colonies obtained from day 8 and 9 heterozygous and homozygous deficient EB was significantly reduced compared to wild-type EB ($p < 0.05$), but by day 10 the number of colonies obtained was greatly reduced but similar from all EB. The number of granulocyte/ macrophage colonies obtained from day 8 homozygous deficient EB was significantly reduced ($p < 0.05$) compared to wild-type, while comparison of day 9 and 10 EB revealed no significant difference. **CONCLUSION**: These results suggest that GATA-4 and GATA-6 transcription factors are not important for hemangioblast formation or primitive hematopoiesis but are necessary for normal definitive hematopoiesis in EB.

2765

**Facilitation of Allogeneic Stem Cell Engraftment Requires a TCRβ-FcRγ Signaling Pathway**

K. N. Taylor, Y. L. Colson; Division of Thoracic Surgery, Department of Surgery, Brigham and Women's Hospital, Boston, MA

The transplantation of purified allogeneic hematopoietic stem cells (SC) fails to establish reliable engraftment capable of rescuing MHC-disparate allogeneic recipients from radiation-induced aplasia. The facilitating cell (FC), a bone marrow-derived cell population, promotes allogeneic SC engraftment without graft vs. host disease (GVHD), thus contributing to the successful reconstitution of the immune system. Biochemical analysis has shown that T cell receptor beta (TCRβ) is present on the FC, but not within the conventional αβTCR complex. Instead, TCRβ is disulfide linked to FCp33, a unique 33kDa protein that is distinct from previously described TCR proteins. Furthermore, FC isolated from TCRβ-deficient mice fail to facilitate engraftment of purified allogeneic SC. Given that *in vivo* FC-mediated SC engraftment is dependent on FcRγ but not CD3ζ, we hypothesized that FC signaling utilizes an FcRγ pathway. Therefore, we investigate the signaling pathway necessary for FC-mediated SC alloengraftment. The current study demonstrates that FcRγ gene and protein expression is significantly greater than CD3ζ expression within the FC as evident by real-time PCR and western blot analysis of immunoprecipitated proteins. Furthermore, the dominant FcRγ protein expression within the FC suggests that FcRγ is associated with the TCRβ complex in these cells. To confirm this, TCRβ immunoprecipitation was performed and revealed the presence of FcRγ and Fcp33 in the co-precipitate, thus demonstrating the association of FcRγ with the unique TCRβ-Fcp33 signaling complex. Furthermore, in vivo studies showed that CD16, an FcRγ-dependent receptor, was not required for FC function. In conclusion, unlike mature $TCR\beta^+$ T cells that utilize CD3ζ signaling and elicit alloreactive responses, FC facilitation of SC alloengraftment without GVHD is mediated through the TCRβ-FCp33 receptor complex via an FcRγ-dependent signaling pathway. These findings provide the first evidence that TCRβ-FcRγ signaling pathways are important in allogeneic SC reconstitution.

2766

**Isolation and Characterization of Myogenic Stem Cells from Adult Murine Skeletal Muscle and Bone Marrow**

Z. Qu-Petersen; The Copenhagen Muscle Research Centre and Department of Neurology, Copenhagen National University Hospital, Copenhagen, Denmark

Skeletal muscle contains a stem cell pool of committed and pluripotent myogenic cells responsible for the growth and repair of postnatal muscle tissue. Investigations suggest that the pluripotent myogenic stem cells (pMySCs) can be of muscle and haematopoietic origins. To identify the pMySCs in different sources, we recently isolated distinct populations of MySCs from skeletal muscle and bone marrow of C57 BL/6 normal mice (5-7 weeks old) by preplate and clonal culture technique. Both populations are clonogenic, capable of extensive proliferation, and multi-lineage differentiation, demonstrating their property of stem cells. By flow cytometry, the muscle-derived MySCs (m-MySCs) were phenotypically characterized as Sca-1(+)CD34(-)CD24(-) while the bone marrow-derived MySCs (bm-MySCs) were characterized as Sca-1(+)CD34(low)CD24(+). Following initial differentiation in culture, both populations displayed a high degree of myogenicity, with 86% of m-MySCs and 64% of bm-MySCs capable of committing into myogenic lineage, but different myogenic pathways were identified. The m-MySCs generated three major progenies concerning specific neurons, glial cells and myoblasts, all of which contributed in vitro to multiple-myotube formation. The roles of the different myotubes (neuron/glia-reated or myoblast-related) in muscle development and regeneration should be investigated further. In contrast, only myoblast-related myotube formation was observed with bm-MySCs in culture. However, the capacity of bm-MySC's clonal-proliferation and robust myofiber formation both in vitro and over transplantation suggest their potential use in muscular disease. In conclusion, the bm-MySCs represent a population of more committed myogenic stem cells in hematopoietic system, while the m-MySC represent a novel class of pluripotent neuromuscular progenitors/stem cells within skeletal muscle.

2767

**Identification of a Novel Monoclonal Antibody to a Cell Surface Antigen that Isolates Neuronal Stem Cells From Murine and Pig Adult Skeletal Muscle and Murine Embryo**

S. O. Winitsky, T. V. Gopal, S. Hassanzadeh, Y. A. Kassahun, N. D. Epstein; NHLBI, NIH, Bethesda, MD

We have previously reported a novel population (named SPOC cells) of non-satellite, non-hematopoietic, cells in adult murine skeletal muscle that differentiate into cardiac myocytes in vitro and in vivo (PLOS, April, 2005). We now report a novel monoclonal antibody (MP804), raised from the total SPOC cell isolate, which when used in conjunction with Sca1, separates the fresh undifferentiated SPOC cells into 3 distinct classes that mature in vitro into either cardiomyocytes, adipocytes or cells with neuronal markers and morphology. The $Sca1^{-}/MP804^{+}$ cells, grown in isolation retain the 804 antigen from their initial undifferentiated state through their differentiation into newly mature β-3 tubulin positive neuroepithelial cells. When not cultured in isolation from the complement of SPOC cells, the neuronal phenotype is suppressed. The 804 antigen is restricted to a subset of cells in murine olfactory lobe, hippocampus and the subependymal layer of the lateral ventricle, the 3 known sites of neuronal stem cells. The 804 antigen is conserved in pig and human. It appears first, in the day 12.5 mouse embryo. Culturing these $804^{+}$ embryonic cells produces the same β-3 tubulin positive cells as obtained from adult murine skeletal muscle. Typical neurospheres with $804^{+}$ centers and β-3 tubulin positive cells at the periphery can be cultured as well, from adult pig skeletal muscle SPOC cells. MP804 has been used to immunoprecipitate the antigen. Mass Spectroscopy shows that 804 antigen co-precipitates with the NMDA receptor in hippocampus, but not peripheral brain. Further identification of the 804 antigen is underway. This represents to our knowledge, the first neuronal stem cell antigen suitable for stem cell detection and isolation.

2768

**Satellite Cells from Neonatal Rat Muscle Co-express Multiple Myosin Heavy Chain Isoforms In Vitro**

J. M. Churko, P. A. Merrifield; Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada

Satellite cells (SCs) are myogenic stem cells, located beneath the basal lamina, which play an important role in postnatal skeletal muscle growth and regeneration. SCs can be identified and isolated based upon the expression of markers such as Pax7 and M-cadherin and are heterogeneous with respect to proliferation kinetics and cell fate. Some activated SCs progress to form differentiated muscle fibers while others maintain a stem cell phenotype, and there is controversy as to whether the fiber type potential of SCs is predetermined. For example, mouse SCs express a fast or slow myosin heavy chain (MyHC) phenotype in vitro based upon the fiber type from which they were derived (Rosenblatt et al, Differentiation 60, 39. 1996), while cultured human SCs are reported to co-express both slow and fast MyHCs within the same myotube following differentiation, regardless of their source (Bonavaud et al, Neuromuscul Disord. 11, 747. 2001). To examine the pattern of MyHC in rat SCs, we used Dynal immunobeads to isolate M-cadherin positive (Mcad +) SCs from neonatal rat hindlimb muscle and examined MyHC expression in vitro using western blot and immunocytochemical methods. In both mass and clonal density cell culture, Mcad + cells differentiated to form myotubes which co-expressed both fast (mab 212F, MY32) and slow (mab NOQ, 10D10) MyHCs within the same myotube. These results demonstrate that rat SCs - like SCs from human tissue - are uncommitted with respect to fiber type potential and co-express multiple MyHC isoforms in the absence of external cues such as innervation. (Supported by Discovery Grant 105869-02 RGPIN from NSERC).

2769

**Quantitative Characterization of Label-Retaining Cells in the Mouse Mammary Gland**

R. Fernandez-Gonzalez,[1,2] C. Ortiz de Solorzano,[3] M. Barcellos-Hoff[1]; [1]Cancer Biology Department, Lawrence Berkeley National Laboratory, Berkeley, CA, [2]Joint Graduate Group in Bioengineering, UC Berkeley / UC San Francisco, Berkeley, CA, [3]Morphology and Imaging Group, Center for Applied Medical Research (CIMA), Pamplona, Spain

In many tissues, adult stem cells (ASC) have a precise spatial arrangement that determines both the architecture and function of the tissue, supported by a niche of specialized cells. Since fragments from any region of the mouse mammary gland can regenerate fully functional tissue when transplanted into parenchyma-free mammary stroma, we hypothesized that ASC are homogeneously and randomly distributed along the mammary ductal tree. To test this, we developed an image analysis tool for quantitative characterization of the spatial distribution of cell populations relative to the morphological features of the gland. The input for this tool is the location of epithelial nuclei and their immunostaining profile. Since there are no unique markers for mammary ASC, we studied long-term, label-retaining cells (LRC), which are slow-cycling cells supposed to contain a subpopulation of ASC. Young mice were labeled for 2 weeks with continuous bromodeoxyuridine infusion, followed by a 9-

week chase. LRC were identified in serial sections using antibodies against bromodeoxyuridine-substituted DNA, and their locations were recorded relative to the morphological structures. LRC frequency was higher in large vs. small ducts, and in areas proximal to the nipple vs. distal ones. Spatial analysis indicated that LRC are randomly distributed in smaller ducts, while in large ducts an LRC tends to be next to another one, i.e., they show up in small clusters. To test whether hormone receptor positive cells form a niche for LRC, we co-localized LRC and estrogen receptor α (ER+). Although distribution analysis indicated the independence of the single-labeled populations, a third of the LRC were ER+. This seems to indicate that ER+ cells are not a key component of a potential *niche* around LRC, but they may mark an LRC subpopulation. Supported by DOD BCRP 17-03-1-0594 predoctoral fellowship to RFG.

2770

**The Characteristics of an Established Clonal Murine Intestinal Epitheliocyte (MIE)**

T. Kanaya,[1] H. Aso,[1] K. Miyazawa,[1] T. Minashima,[1] T. Kido,[1] K. Tahara,[2] T. Yamasaki,[2] K. Watanabe,[1] S. Ohwada,[1] T. Yamaguchi[1]; [1]Cellular Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [2]R & D Center, BML Inc., Saitama, Japan

In murine small intestine, cells differentiate into one of five functional cell types, such as enterocytes, goblet cells, enteroendocrine cells, Paneth cells and M cells. All of these cells arose from intestinal epithelial stem cells located at the bottom of crypt. However, the differentiation processes of these cells have not been elucidated. To clarify the differentiation mechanisms of these intestinal epithelial cells, we attempted to establish clonal murine intestinal epithelial cell lines. The small intestine was removed from a C57BL/6 mouse and cut finely, and then seeded into a collagen-coated flask. After limiting dilution, we have established three clonal cell lines. These established clones of murine intestinal epitheliocyte (MIE) are able to grow constantly and assume a monolayer, cobblestone and epithelial-like morphology, with close contact between cells. All of three clones were strongly positive for cytokeratin, a marker for epithelial cells. One clone was selected upon the growing rate. The growth curve of MIE cells showed that cells grew to confluence by 5 days after seeding. Scanning electron microscopy showed that MIE cells formed microvilli tightly after 15 days of culture. Cell-cell adherens junction protein (beta-catenin) and tight junction proteins (ZO-1 and claudin 1) were observed in the cell-cell contact region of adjacent cells. Musashi-1 antigen and hes 1 transcriptional factor are known to be candidate markers for stem cells and early progenitor cells of intestinal epitheliocyte, which are restricted to the bottom of crypt. As MIE cells expressed Musashi-1 and hes 1, this cell line may have characteristics of immature intestinal epithelial cells. MIE cells can contribute to the elucidation of intestinal epithelial cell differentiation mechanisms.

2771

**Papilla: A Niche for Human Renal Stem Cells**

T. Roitbak, E. Romero, A. Wandinger-Ness; Pathology, University of New Mexico, Albuquerque, NM

Many genetic, immunologic and environmentally caused renal disorders lead to kidney damage or loss. As a result, approximately 58,000 patients in the US are currently on the waiting list for a kidney transplant. Despite the advances in kidney transplantation, a significant shortage of donor organs requires many to remain on dialysis for extended periods of time. The role of stem cells in the treatment of pathophysiological disorders is therefore of significant interest. Until recently, the population of adult SC in kidney had not been identified. Based on published studies in rodents (Oliver et al., J Clin Invest. 2004, 114:795-804) we isolated and characterized a population of human primary kidney cells with the characteristics of adult renal stem cells. The cells were derived from the renal papilla of two independent patients. Ultrastructural analyses show these cells reside in situ exclusively in the loops of Henle of tubules found deep within the medulla and papilla. The cells are harbored between the tubular epithelia and readily associate with cortical tubular epithelia in vitro, both on filter cultures and in 3D collagen gel cultures. These putative adult human renal stem cells express numerous markers characteristic of stem cells, are able to adopt tubular epithelial-like and neuronal-like phenotypes, and in some cases form structures resembling neurospheres. Work is in progress to test their ability to incorporate into renal tubules of animal models. The experiments suggest that human papilla may serve as a niche for human renal stem cells and the results are important for the development of tissue engineering and alternate renal tissue repair strategies. Supported by UNM SOM RAC grant.

2772

**Calcium Oscillations in Liver Stem Cells are Associated with Their Acquistion of a Cardiac Phenotype**

B. J. Muller-Borer,[1] W. E. Cascio,[1] G. L. Esch,[2] G. Butz,[2] H. Kim,[2] W. B. Coleman,[2] J. W. Grisham,[2] P. A. W. Anderson,[3] N. N. Malouf[2]; [1]Division of Cardiology, ECU Brody School of Medicine, Greenville, NC, [2]Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, [3]Duke University Medical Center, Durham, NC

We examined in a cardiac microenvironment the plasticity of a liver stem cell line (WB F344) generated from a cloned, single, non-parenchymal epithelial cell from a normal adult male rat. Our previous studies suggested that WB F344 cells acquire a cardiac phenotype in the absence of fusion. Here, we begin investigating the mechanisms that induce these stem cells to acquire a cardiac phenotype. Neonatal cardiac myocytes were co-cultured with WB F344 cells. Intercellular communications were documented with Fluorescent Recovery After Photobleaching. Line scan confocal images recorded intracellular calcium signals. Real-time RT-PCR and immunocytochemistry monitored transcription of cardiac specifying transcription factors and expression of cardiac specific proteins. De novo oscillating $[Ca2+]_i$ spikes in the cytoplasm of WB F344 cells identified 24-36 hours after co-culture were synchronous with oscillating $[Ca2+]_i$ transients in adjacent cardiomyocytes. These were associated with transcription of NKx2.5, MEFc2, myocardin, Tbx5 and Tbx20 in the WB F344 cells. Differentiated WB F344-derived myocytes, expressing cardiac TnI and TnT in sarcomeres appeared 4-5 days later. A physical contact between the myocytes and the WB F344 cells was a requirement for the acquisition of $[Ca2+]_i$ spikes and eventually a cardiac phenotype. Intercellular communications between the two cell types, both expressing connexin 43, was achieved through shared gap junctional channels. Uncoupling the cells with carbenoxolone extinguished the $[Ca^{2+}]_i$ oscillations in the WB F344 cells but not those in the myocytes. Following removal of carbenoxolone, the WB F344 cells reacquired $[Ca2+]_i$ oscillations. We hypothesize that a signal, presumably transmitted via gap junctions into the WB F344 cells, resulted in oscillating calcium signals that were transduced into transcription of a cardiac specifying gene program and the acquisition of a cardiac phenotype in the WB F344 cells.

2773

**Expansion of Hepatocytes in Mitomycin C Treated DPPIV Deficient Fischer Rats**

D. C. Hixson,[1,2] M. Carreiro,[1] R. Simper Ronan,[1] K. Brilliant[1]; [1]Department of Medicine, Rhode Island Hospital, Providence, RI, [2]Medicine and Pathology (Research), Brown University School of Medicine, Providence, RI

Compromising regeneration of donor hepatocytes by pretreatment of host rats with retrorsine has been widely used to promote selective expansion of transplanted hepatic progenitor cells. Retrorsine crosslinks DNA of host hepatocytes and prevents their proliferation in response to partial hepatectomy (PH), allowing selective expansion/differentiation of transplanted progenitors. Two shortcomings of this method are the length of the protocol (7 weeks) and the carcinogenicity of retrorsine, an activity that precludes human usage. In this report, we describe a clinically applicable protocol for expansion of transplanted progenitor cells that uses a one week pretreatment with Mitomycin C, a well characterized chemotherapy agent. To determine the efficacy of Mitomycin C, mutant Fischer 344 rats expressing inactive dipeptidyl peptidase IV (DPPIV) were injected i.p. with Mitomycin C (2 mg/kg). Seven days after treatment, host rats were given a 2/3 PH, injected intrasplenically with adult or fetal hepatocytes from DPPIV+ Fischer 344 rats followed by i.p. injection of antibiotics (5mg/kg) starting one day post-surgery. Histochemical staining of frozen sections from host rat livers for DPPIV enzymatic activity revealed well-defined colonies of donor derived hepatocytes by 2 weeks post transplantation. Comparative studies showed similar levels of engraftment in host rats treated with Mitomycin C and retrorsine. The size distribution of DPPIV+ donor hepatocyte colonies determined with ImageProPlus software was comparable in retrorsine and Mitomycin C treated host rats. Primary advantages of this protocol are the rapid production of donor colonies and the possibility of developing a clinically applicable protocol for repopulating a diseased liver with adult or fetal hepatic progenitor cells.

2774

**Differentiation of Pluripotent Dental Pulp Stem Cells into Osteoblastic Lineage**

T. Nozaki, M. Shinohara, K. Ohura; Department of Pharmacology, Osaka Dental University, Osaka, Japan

**Objective**: To evaluate the potency of dental pulp stem cells as a source of osteoprogenitor cells by investigating the potential of rat dental pulp stem cells to differentiate into an osteoblastic lineage *in vitro*. **Methods**: Rat dental pulp stem cells were induced to differentiate using conditions reported to induce osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. Nodule mineralization and alkaline phosphatase (ALP) activity were measured after the induction. Quantitative analysis of gene expression was performed using real-time RT-PCR to elucidate gene regulation in the potential osteoblastic lineage obtained. **Results**: Low levels of mineralization were detected at 3 weeks after induction, and this increased to 29 mineralized nodules at 6 weeks, as determined by von Kossa staining. The ratios of ALP-positive cells among the total cells were 44.4±1.0, 46.9±0.9, 40.5±0.5 and 21.0±1.8% at 1, 2, 3 and 6 weeks after induction, respectively, and the level of ALP activity was significantly decreased at 6 weeks ($p<0.05$). At 6 weeks after induction, Col1a2 (collagen type I) and Bglap (osteocalcin) were up-regulated by approximately 1.7-fold and 1.3-fold, respectively, compared with their corresponding levels at 1 week. These observations suggest that the rat dental pulp stem cells differentiated into osteoblastic cells and that the ALP activity decreased in parallel with the differentiation. Moreover, the expressions of Col1a2 and Bglap were inversely correlated with Alpl (alkaline phosphatase) expression after the induction, whereas Bmp2 (bone morphogenetic protein 2) expression showed a positive correlation with Alp1 expression. **Conclusions**: The present results suggest that rat dental pulp stem cells are able to differentiate into an osteoblastic lineage, and provide support for the hypothesis that mesenchymal stem cells in rat dental pulp may represent a source of osteoprogenitor cells that could function in clinically relevant osteogenic regeneration.

2775

**Oct-4-GFP Imaging of Cloned and Fertilized Mouse Embryos at the Morula Stage Predicts Success of Stem Cell Derivation**

P. J. Sammak,[1] F. Cavaleri,[2] H. Schöler,[2] G. Schatten,[1] M. Boiani[2]; [1]Pittsburgh Development Center, University of Pittsburgh, Pittsburgh, PA, [2]Max Planck Institute for Molecular Biomedicine, Muenster, Germany

Neither reproductive nor stem cell potentials of individual embryos can be accurately predicted, notwithstanding the importance of potentiality for biomedical research as well as reproductive medicine. Here, real-time Oct4-GFP detection is shown as a quantitative method for predicting developmental competence of mouse embryos generated by fertilization and by nuclear transfer (NT) for both ESC derivations as well as for pregnancies. Real-Time Spinning Disc Confocal Microscopy, with precise temperature and gas regulation, detects *Oct4* gene reprogramming in NT and fertilized mouse embryos. Embryos were observed in time lapse during development. Day 2 embryos began expressing Oct4-GFP at the 4-cell stage and became progressively, but not uniformly brighter within the next 10 hrs. In day 3-4 embryos, cells that were destined to become the inner cell mass could be identified within morulae by two characteristics, brightness and number of bright cells in apposition, before embryo compaction and cavitation. Blastomere quality was predicted during the morula stage and morulae were sorted into three groups, based on Oct4-GFP intensity and localization to the nascent inner cell mass (ICM), to evaluate the predictive value of this non-invasive imaging before blastocyst formation. Blastocyst development (type 1, 68%; type 2, 58%; type 3, 38%), ESC derivation rates cells (36%, 27%, 9%), and germ-line transmission of ESC (28%, 38%, 16% $GFP^+$ gonads) all demonstrate this method's power. 50% of NT-embryos developed to blastocysts and 25% established ESC lines, although only 1% develop to offspring. Higher order patterns of pluripotency markers within embryos are correlated to ES derivation success, providing a biologically-based distinction between NT-constructs with negligible reproductive potentials from those invaluable for therapeutic cloning.

2776

**Comparative Analysis Between Diploid & Tetraploid Stem Cell - Derived Teratomas Using MRI and Histopathology**

C. A. Castro,[1] P. H. Mills,[2] J. H. Park,[3] J. A. Ozolek,[1] E. T. Ahrens,[2] G. P. Schatten[1]; [1]Pittsburgh Development Center, University of Pittsburgh, Pittsburgh, PA, [2]Biological Sciences, Carnegie Mellon, Pittsburgh, PA, [3]Medical Research Center, MizMedi Hospital, Seoul, Republic of Korea

Diploid and tetraploid monkey stem cell teratomas were grown for analysis and comparison. Stem cells were bilaterally injected in the testes of mice and allowed to grow for approximately 10 weeks. After growth, the teratomas were removed for 3-D MRI imaging and histopathological sectioning. Samples of the teratomas were taken fresh for karyotype confirmation and tissue was also frozen and stored for possible future DNA and RNA analysis. To non-invasively acquire volumetric tissue information and identify structures, each teratoma was positioned in an 11.7T microMRI system for imaging. Each image set was imported into 3-D scientific software for the browsing of serial images along the axial, sagittal

and coronal axes and the calculation of volumetric tissue statistics. Afterwards, the teratomas were serially sectioned in the long axis and stained using H&E and a battery of immunostains. The slides produced were analyzed to compare the microanatomy between the diploid and tetraploid teratomas. Also, serial sections were compared with the MRI "virtual histology". The tetraploid teratomas appear to have grown faster than the diploid. Both types presented three germ layers but in different percentages. The tetraploid teratomas appear to be less mature and less differentiated, and also more necrotic, apoptotic, mitotic, and disorganized. In addition, tetraploids appear to be more malignant with atypical mitosis, nuclei and cytoplasmic atypia and less organogenesis capabilities than the diploids. Histopathology and MRI shows that the anatomy of the tetraploid teratoma in general has the tendency to be abnormal while the anatomy of the diploid teratoma is more likely to be normal.

2777

**Germline Stem Cells in *C elegans***

S. L. Crittenden,[1] K. A. Leonhard,[2] D. T. Byrd,[2] J. Kimble[1,2]; [1]Howard Hughes Medical Institute, Madison, WI, [2]Department of Biochemistry, University of Wisconsin-Madison, Madison, WI

The adult *C. elegans* germ line includes mitotically-dividing cells in the distal "mitotic region" and meiotic cells more proximally. A single somatic cell, the distal tip cell (DTC), provides a "niche" that promotes germline mitoses by GLP-1/Notch signaling. Downstream of GLP-1/Notch signaling, PUF RNA regulators promote mitosis by repressing meiosis-promoting GLD RNA regulators. We have investigated the self-renewal and division patterns of cells in the mitotic region. To investigate self-renewal, we labeled cells in the mitotic region with BrdU. Within 10 hours all mitotic germ cells are labeled. In a series of pulse/chase experiments, BrdU is lost from the mitotic region and BrdU labeled nuclei move into the proximal meiotic region. Therefore, cells in the mitotic region are capable of both self-renewal and generation of differentiating cells, both hallmarks of stem cells. The position of nuclei within the mitotic region does not affect the rate at which they lose BrdU labeling and all nuclei cycle at approximately the same rate. To investigate division patterns, we examined metaphase plates and mitotic spindles throughout the mitotic region, and see no asymmetric or oriented cell divisions. Our current model is that *C. elegans* germline stem cells are controlled by position with respect to the niche rather than by oriented asymmetric cell divisions.

2778

**Interstitial Cells in Reproductive Strategy of Colonial Hydroid *Obelia longissima***

A. V. Akhmadieva, A. I. Shukalyuk, V. V. Isaeva; Laboratory of Embryology, Institute of Marine Biology FEB RAS, Vladivostok, Russian Federation

In colonial invertebrates the line of totipotent or pluripotent stem cells is maintained throughout the life of the animal, and these self-renewing stem cells may give rise to all cell lines including germ cells. We performed histological, ultrastructural, histochemical and immunochemical studies of stem and gonial cells in the colonial hydroid *Obelia longissima.* Interstitial and gonial cells in *O. longissima* have morphological features typical for stem and germ cells in all studied *Metazoa*: large nucleus with diffuse chromatin and large nucleolus, basophilic cytoplasm including specific germinal bodies, surrounded with mitochondria; in oogenic cells annulate lamellae were also found. Interstitial and germ cells in *O. longissima* selectively express alkaline phosphatase activity, a histochemical marker for mammalian embryonic stem and primary germ cells. The specific deep brick-red color of interstitial and primary germ cells, migrating inside stolons and participating in medusoid formation, is comparable with that revealed in murine ESC *in vitro,* used as a standard. We also used proliferating cell nuclear antigen (PCNA; DAKO, USA) to detect self-renewing cells that have not left mitotic cycle. Stem and primary germ cells in *O. longissima* were PCNA-positive while differentiated somatic cells were PCNA-negative. Our findings show that properties of interstitial and germ cells in the colonial hydroid *O. longissima* is ensured by evolutionary conservative mechanisms, common for all studied invertebrate and vertebrate animals from cnidarians to mammals. A self-renewing pool of interstitial cells in the colonial hydroid provides the cell source for asexual and sexual reproduction and the cellular basis for realization of the life strategy including both alternating reproductive modes.

2779

**Stem and Germ Cells in Asexually Reproducing Animals: A Comparative Study**

A. I. Shukalyuk, V. V. Isaeva; Laboratory of Embryology, Institute of Marine Biology FEB RAS, Vladivostok, Russian Federation

A self-renewing pool of totipotent or pluripotent SC provides the cell source for asexual and sexual reproduction in colonial invertebrates or those capable of asexual reproduction. Our study provides a step forward in the elucidation of evolutionary conservative sub-cellular and molecular bases of "stemness," focusing on the comparative study of stem and germ cells in colonial rhizocephalan crustaceans, cnidarians, planarians, using different methodological approaches. Stem and germ cells of all studied animals have typical germinal bodies surrounded with mitochondria. Our data provide evidence of mitochondria involvement in biogenesis of germ determinants; the release of mitochondrial matrix in cytoplasm of gonial cells in cnidarians, turbellarians, echinoderms and vertebrates supposedly mediates the transport and incorporation of mitochondrial derivatives into germ determinants. These findings agree with data of other authors on the presence of mitochondrial ribosomal RNA in germ determinants. SC of colonial rhizocephalans and of a hydroid selectively express activity of alkaline phosphatase, a histochemical marker for mammalian embryonic SC and GC. We carried out immunocytochemical tests using proliferating cell nuclear antigen to detect self-renewing cells that have not left the mitotic cycle in stolons of the rhizocephalan *P. gracilis* and cnidarian *O. longissima*. Only stem and germ cells were PCNA-positive while differentiated cells were PCNA-negative. We found the evolutionary conservative gene *vasa* in the DNA of studied rhizocephalan crustaceans. We cloned *vasa*-related genes and other genes of DEAD-box family from the colonial rhizocephalan *Polyascus polygenea,* and phylogenetic analysis suggest that these genes are closely related to *vasa*-like genes of other Arthropoda. We found selective expression of *vasa*-like gene products in SC and GC in *P. polygenea*. Further studies of SC in a wide variety of metazoans may provide a clearer understanding of common, evolutionary conservative bases for totipotency and immortality.

## Endosomes & Lysosomes II (2780-2795)

2780

### Autophagy Pathway Is Involved in the Degradation of Hirano Bodies

D. Kim, M. Fechheimer; Cellular Biology, University of Georgia, Athens, GA

Hirano bodies are actin-rich aggregates that have been found in a number of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis, and other pathological conditions. The fate and mechanism of degradation of Hirano bodies are poorly understood. Here, we show that autophagy, a process mediating bulk degradation of cytoplasmic proteins or organelles, is involved in the degradation of Hirano bodies. The formation of Hirano bodies was controlled by regulated inducible expression of the 34 kD ΔEF1 protein in wild type or autophagy mutant strains of *Dictyostelium*. The growth rate of a *Dictyostelium* mutant strain (ATG $5^-$) with Hirano bodies was severely reduced up to 72hrs when compared to that of wild type *Dictyostelium* bearing Hirano bodies or autophagy mutant without Hirano bodies. We also observed the co-localization of atg8-GFP, an autophagosome marker protein, with Hirano bodies by fluorescence microscopy. Measurements of the rate of degradation of 34kDa ΔEF1-GFP protein in both wild type and autophagy mutant *Dictyostelium* were performed using flow cytometry and western blot analyses. The turnover of Hirano bodies is faster in wild type than in autophagy mutant (ATG $5^-$) *Dictyostelium* and the background expression level of 34kDa ΔEF1-GFP protein is significantly higher in autophagy mutant (ATG $5^-$) than in wild type *Dictyostelium*. These results suggest that autophagy is involved in the degradation of Hirano bodies. Yet, significant turnover of Hirano bodies still occurs in ATG$5^-$ cells, suggesting that other mechanisms such as ubiquitin-mediated degradation also contribute to turnover of Hirano bodies. Since failure of clearance of Hirano bodies by autophagy blocks cell growth in *Dictyostelium*, it is likely that degradation of Hirano bodies by autophagy may promote cellular function and survival during the progression of neurodegenerative disease.

2781

### Phosphatidylserine in Addition to Phosphatidylethanolamine is *in vitro* Target of the Mammalian Atg8 Modifiers, LC3, GABARAP And GATE-16

I. Tanida, Y. Sou, M. Komatsu, T. Ueno, E. Kominami; Department of Biochemistry, Juntendo University, School of Medicine, Tokyo, Japan

In yeast, phosphatidylethanolamine is a target of the Atg8 modifier in ubiquitylation-like reactions essential for autophagy. Three human Atg8 homologs, LC3, GABARAP and GATE-16, have been characterized as modifiers in reactions mediated by hAtg7, an E1-like enzyme, and hAtg3, an E2-like enzyme, as in yeast Atg8-lipidation, but their final target(s) has not been identified. Recently, results from the incubation of [$^{14}$C]ethanolamine in COS7 cells for 48 h has suggested that phosphatidylethanolamine is a target of LC3. These results are not conclusive, however, because of the long incubation time. To identify the phospholipid target(s) of the Atg8 homologs, we reconstituted the conjugation systems for the mammalian Atg8 homologs *in vitro* using purified recombinant Atg proteins and liposomes. Each purified mutant Atg8 homolog with an exposed C-terminal Gly formed an E1-substrate intermediate with hAtg7 *via* a thioester bond in an ATP-dependent manner, and formed an E2-substrate intermediate with hAtg3 *via* thioester bond dependent on ATP and hAtg7. When each Atg8 homolog was incubated with liposomes composed of the total lipids of HeLa cells, its conjugated form was observed in the presence of hAtg7, hAtg3 and ATP. *In vitro* conjugation using synthetic phospholipid liposomes showed that phosphatidylserine in addition to phosphatidylethanolamine is a target of LC3, GABARAP, and GATE-16. To our knowledge, this is the first report showing that phosphatidylserine is a target of ubiquitin-like modifiers.

2782

### Gold-Containing Endosome-Derived Vesicles that Cannot Fuse with Autophagosomes

C. Balane, J. Wolfe; Biology, Wesleyan University, Middletown, CT

Autophagy of the parental macronucleus occurs during the sexual life cycle of the unicellular ciliate *Tetrahymena thermophila*. The macronucleus undergoes massive compaction, acidification, and rapid degeneration. Nuclear acidification suggests lysosomal mediation, and therefore autophagy. In addition, acid phosphatase, a lysosomal enzyme, and the fluorescent lysosomal marker Texas Red Dextran (TRD) co-localize with degrading macronuclei. Such co-localization is impeded by autophagic inhibitors 3-Methyl Adenine and Bafilomycin A. In addition, both inhibitors prevent macronuclear loss, suggesting that TRD co-localization with the macronucleus corresponds to hydrolytic enzyme transfer. To monitor macronuclear autophagy by electron microscopy, cells were incubated with 6 nm colloidal gold together with TRD. Intracellular gold was visualized by silver enhancement. We expected that gold would co-localize with the degrading macronucleus. To our surprise, TRD became co-localized with degrading macronuclei, but the gold particles never did. We infer that although TRD and gold enter cells together, during the course of endosomal processing, two types of vesicles are formed, one of which excludes gold. This was confirmed cytologically; some vesicles contained both gold and TRD, while others contained TRD alone. We next tested sorting in cells whose lysosomes were loaded with TRD and an alternative marker for electron microscopy, cationized ferritin. Preliminary experiments indicate that fluoresceinated ferritin co-localizes with TRD in vesicles derived from the primary endosome. It was also observed that ferritin co-localizes with degrading macronuclei, together with TRD. Evidently, only gold-free vesicles can fuse with autophagosomes, while gold-positive vesicles cannot. One possibility relates to the non-organic nature of gold, another to its large size, a third to its density. It will be possible to distinguish among these alternatives using colloidal gold coated with protein, as well as particles of differing sizes and composition.

2783

### Phagosome/Lysosome Fusion is Preceded by an Actin and Calmodulin-Dependent Docking Step

W. Stockinger, L. Jarzylo, E. Shieh, A. B. Castoreno, A. Nohturfft; MCB, Harvard University, Cambridge, MA

Particles greater than 0.5 µm in diameter such as microbes, apoptotic bodies and retinal outer segment fragments are often eliminated through an endocytic process called phagocytosis. Inside cells particles are stored in specialized vacuoles termed phagosomes. Phagosomes fuse with vesicles of the endosomal/lysosomal system, which allows for delivery of hydrolytic enzymes and eventually for the digestion of the engulfed material. In an effort to study the process of lysosome/phagosome fusion in biochemical detail we have developed a novel cell-free assay. In this assay tritium-labeled lysosomes are mixed with phagosomes containing scintillant latex beads. As the effective range of tritium-derived beta particles is less than 600 nm, scintillation requires close interaction between donors and acceptors. We found that the assay produces a signal in a cytosol, ATP and

temperature-dependent manner. When samples are subsequently treated with carbonate the signal is abolished, indicating that the vesicles have docked but not fused. When vesicles are allowed to dock and then exposed to $Ca^{2+}$, a complex is formed that remains resistant to carbonate. Through the use of inhibitors and through chromatographic fractionation of cytosol we show that docking requires actin polymerization and calmodulin. The results were confirmed by immunofluorescence microscopy and with a live-cell scintillation proximity assay. These findings offer new insights into the mechanisms of phagosome maturation and provide a useful new system for further biochemical dissection.

2784

**Induction of Macroautophagy by Proteasome Inhibition in Living Cells**

D. W. Hailey, E. L. Whiteman, H. Lorenz, J. Lippincott-Schwartz; NIH / NICHD / CBMB, Bethesda, MD

Macroautophagy is an inducible process by which cytosolic components are sequestered in double-membrane bound vesicles and delivered to lysosomes for destruction. Increased numbers of autophagosomes are observed in some neurodegenerative disorders including Alzheimer's and Huntington's disease. Autophagosomes may play a protective role in these diseases by isolating and degrading misfolded or aggregated proteins. Suppression of proteosome activity is observed in many of these same neurologic diseases. We asked whether loss of proteosome activity can induce macroautophagy, and if so, what mechanisms regulate it under these conditions. We created stable cell lines expressing the mature autophagosome marker CFP-Atg8. Under growth conditions, we observe fewer than 10 autophagic vesicles per cell. These structures contain authentic substrates of macroautophagy (RFP-GAPDH and YFP-LDH). To mimic conditions of reduced proteosome capacity, we treated cells with low concentrations of proteosome inhibitors. This treatment dramatically increases the number of autophagic vesicles in a dose and time dependent manner, and CFP-Atg8 protein levels concomitantly increase. Concurrently, freely diffusing CFP-Atg8 is sharply reduced via its recruitment to membranes. This effect is abolished by addition of 3-methyladenine. Saturation of the 26S proteosome, effected by overexpression of CD3δ-YFP, similarly induces the appearance of autophagosomes. Aggregations of CD3δ-YFP in the ER membrane also colocalize with CFP-Atg8. Last, we studied the effect of proteosome inhibition on a marker for nascent autophagosomes, GFP-Atg5. We found that the mobility of GFP-Atg5 is dramatically reduced under these conditions, suggesting that loss of proteosome activity affects biogenesis of autophagosomes at an early step. Our data suggests a feedback mechanism in which impaired proteosomal degradation upregulates levels of critical component proteins of the autophagosome. We propose that autophagic induction may be stimulated by increasing the levels of these proteins and by recruiting autophagic machinery to proteosomal substrates.

2785

**Mycobacteria Escape from Phagosomes**

P. J. Peters,[1] N. van der Wel,[1] D. Houben,[1] D. Hava,[2] D. Fluitsma,[3] M. Brenner[2]; [1]Netherlands Cancer Institute, Amsterdam, The Netherlands, [2]Harvard Medical School, Boston, MA, [3]Free University, Amsterdam, The Netherlands

In macrophages, mycobacteria are assumed to reside in early endosomes. In the endocytic pathway MHC class II and CD1 antigen loading is facilitated while MHC class I loading of cytosolic antigens occurs in the ER. Since T-cells are known to be involved in the immune response to mycobacteria infections, the localisation of mycobateria was determined in both macrophages and dendritic cells in relation to the localization of the antigen presenting molecules. M. tuberculosis and M. leprae infection was monitored at different infection periods. Antigen presenting molecules MHC class II and CD1b but also LAMP1 were after infection, transiently detected on the phagosomes. No ER fusion with the phagosome was seen using a immunogold label quantification with ER resident proteins*. However detailed analysis using cryo-immunogold electron microscopy revealing all membranous compartments, demonstrated that after 48 hours of infection, the bacteria without LAMP labeling also missed the electron lucent space normally detected in the phagosome and importantly, were not enclosed by a host cell membrane. These cytosolic bacteria were detected in increasing numbers after 92 hours of infection. In conclusion, the trafficking of mycobacteria appears to involve more compartments then previously reported. The mycobacteria traffic through all compartments, which allow access to MHC class I, class II and CD1 presentation. The presence of mycobateria in the cytosol of skin and cultured cells, explains the MHC class I restricted CD8 T-cells involved in resistance to mycobacteria that was long a phenomenon not fully understood. * Touret et al., Cell 2005 in press

2786

**A Novel Method to Purify Phagosomes Demonstrates a Modulation of Phagosomal Maturation by *Streptococcus Pyogenes* Bacteria**

P. Nordenfelt, P. Lönnbro, H. Tapper; Department of Clinical Sciences, Clinical and Experimental Infectious Medicine, Lund, Sweden

*Streptococcus pyogenes* can cause severe and potentially lethal infections by evading the host cellular immune system. In order to study the interaction between *S. pyogenes* and human phagocytes, we have developed a novel method for phagosome isolation using ATRA-differentiated neutrophil-like HL-60 cells and magnetic bead-coupled AP1 (wild-type) and BMJ71 (mutant, lacking M and H surface proteins) strains of *S. pyogenes* bacteria. After nitrogen cavitation of phagocytosing cells, pure phagosome preparations were obtained by magnetic separation. Isolated phagosomes at different stages of maturation were analysed by Western blot for the presence of the bactericidal protein myeloperoxidase (MPO), and we could demonstrate a difference in the maturation pattern between phagosomes containing wild-type and mutant streptococci. For the wild-type AP1 strain, phagosomal content of MPO diminished over time, whereas it increased for phagosomes containing the mutant BMJ71, lacking proteins M and H. Our novel method of phagosome isolation could be applied to the study of manipulation of phagosomal maturation also by other intracellular pathogens.

2787

**Analysis of Anti-Protease Activity in Dendritic Cell Lysosomes**

G. Baravalle, E. S. Trombetta, N. McCurley, I. Mellman; Department of Cell Biology and Section of Immunobiology, Yale University School of Medicine, New Haven, CT

Dendritic cells (DCs) are the immune system’s most potent antigen-presenting cells. Upon appropriate stimulation immature DCs, which are specialized for antigen capture, transform into mature cells that specialize in the presentation of antigenic peptide-MHC complexes (pMHC). This, in turn, leads to T cell stimulation. Despite these hallmark signs of DC maturation, the mechanisms that regulate their capacity for antigen processing and transportation of pMHC to the cell surface is not completely understood. It has been shown that DCs regulate their proteolytic

activity by a generalized activation of lysosomal function, which results in enhanced lysosomal acidification in mature DCs as compared to immature DCs. This decrease in the lysosomal pH of mature DCs augments antigen proteolysis, and thus results in the efficient formation of pMHCs. Our work on specific lysosomal protease-inhibitors hints at the control of antigen processing at the level of immature DCs. Performing *in vitro* degradation assays using a mixture of cell lysates of CD11c-purified immature or mature mouse bone marrow derived DCs and macrophages, we show that digestion of some proteins was decelerated by a factor contained primarily in immature DC lysates. Since treatment of the DC lysates with proteinase K led to a decrease of this effect, this factor may be a protein. Thus, the ability of DCs to developmentally regulate their capacity for proteolysis is likely not only due to control of lysosomal acidification but also to modulation of anti-protease activity. Supported by the Austrian Science Fund (FWF).

2788

**The Role of Atg9, a Multi-spanning TGN and Endosomal Membrane Protein, in Mammalian Autophagy**

A. R. J. Young,[1] R. Koechl,[1] X. W. Hu,[1] S. G. Crawshaw,[2] S. High,[2] J. Lippincott-Schwartz,[3] D. W. Hailey,[3] S. A. Tooze[1]; [1]Secretory Pathways Laboratory, Cancer Research UK London Research Institute, London, United Kingdom, [2]University of Manchester, Manchester, United Kingdom, [3]NIH / NICHD / CBMB, Bethesda, MD

During amino acid depravation cells undergo autophagy order to maintain homeostasis. Autophagosomes (AVs), autophagic vesicles, appear in the cytoplasm and engulf cytoplasm and organelles. These immature autophagosomes (AVis) fuse with vesicles of the endosomal/lysosomal system, and become degradative AVs, AVds. Their contents are degraded in a low pH, protease-containing environment without adversely affecting the viability of the cell. The origin of autophagic vesicles in mammalian cells is still unclear. There are now known to be between 20 and 30 genes in yeast (Atg, Autophagy genes) many of which are conserved in mammalian cells. We show that the mammalian ortholog of Atg9 localises to the Golgi complex, being enriched in the trans-Golgi network (TGN), and late endosomes, in particular Rab7 and Rab9 containing late endosomes. Mammalian Atg9 (mAtg9) is glycosylated, with the N-terminal domain present in the cytosol. In primary rat hepatocytes or in cell lines, under starvation conditions, a population of mAtg9 was found to localise transiently to AVs containing GFP-LC3. Using subcellular fractionation to isolate AVs from hepatocytes we find mAtg9 is enriched, as is LC3-II, a marker for AVs. Immunogold labelling of cryosections reveals that while a very minor amount of mAtg9 is found on the AV membranes, most is found on internal membranes, presumably engulfed by the AVi during formation. siRNA depletion of mAtg9 reduced the number of AVis in primary hepatocytes after starvation. The localization of mAtg9 changes upon starvation, becoming more dispersed, and less peri-nuclear. Knock down of mAtg1 (ULK-1) by siRNA causes Atg9 to accumulate in a peri-nuclear location. Our data suggest that AVs, nucleated by interaction between mAtg1 and mAtg9 arise from a TGN-derived compartment, which may overlap with Rab9-containing membranes.

2789

**Mechanisms Controlling Polarised Delivery of Secretory Lysosomes to the CTL Immunological Synapse**

J. C. Stinchcombe, G. M. Griffiths; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Cytotoxic T-lymphocytes (CTL) kill infected cells by releasing cytotoxic proteins which induce apoptosis in their targets. CTL have evolved mechanisms to kill targets effectively whilst protecting neighbouring cells. 1) Cytotoxic proteins are stored intracellularly, and only released on contact with a target. Unusually, lysosomes act as secretory organelles in CTL. 2) Exocytosis occurs at a specific site on the cell surface, opposite a gap which forms in the otherwise tight CTL:target membrane contact and creates a sealed cleft into which material is secreted. Thus, released proteins are directed towards the target, but segregated from neighbouring cells. The secretory site is defined by 1) reorganisation of surface proteins into a secretory immunological synapse (ISS) in which adhesion molecules form a ring of cell:cell contact, surrounding the secretory site and a separate signalling domain, and 2) reorientation of the microtubule cytoskeleton (and its associated organelles) towards the target. We are investigating mechanisms of targeted lysosome delivery to the secretory site during killing, using light and electron microscopy. We find the MTOC moves from its steady state perinuclear location, to the cell surface at the centre of the ISS, reorganising microtubules to pass the surface within the secretory domain. Thus lysosomes are delivered to exocytic sites during polarised transport towards the MTOC. Following killing, the MTOC retracts from the surface, preventing further delivery and exocytosis of lysosomes. Therefore, MTOC positioning targets lysosomes to secretory sites, and controls secretion by controlling access of lysosomes to these sites. To determine molecules controlling lysosome secretion we identified genetic diseases showing defects in lysosome secretion, and used microscopy techniques to determine the secretory block. This approach has identified roles for AP3, rab27a, and munc13-4 in polarised movement, microtubule detachment and exocytosis of lysosomes, respectively.

2790

**Special Lipid Domains Found in Reservosomes from *Trypanosoma cruzi***

M. G. Pereira,[1] C. Vargas,[2] C. Sant'Anna,[1] W. De Souza,[1] G. C. Atella,[2] N. L. Cunha-e-Silva[1]; [1]Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Instituto de Bioquimica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Reservosomes are acidic compartments that store nutrients, found in epimastigote forms of *Trypanosoma cruzi.* They were first described as multivesicular bodies; however, further ultrastructural studies characterized reservosomes as single-membrane bound organelles that lack true internal vesicles and contain lipid inclusions dispersed in an electrondense protein matrix. The frontier between lipids and the dense protein matrix may assume the trilaminar aspect of a planar lipid bilayer. Reservosomes freeze-fractured inside intact cells present flattened lipid inclusions, similar to those usually found in reservosome ultrathin sections, as well as round inclusions, resembling internal vesicles. We have isolated reservosomes and shown that whole purified organelles present twice more lipids than proteins, cholesterol and ergosterol being their most important neutral lipids (Cunha-e-Silva *et al*, FEMS Microbiol Lett, 2002, 214: 7). Now we aim to describe the lipid composition of their purified membranes, analyze the existence of lipid subdomains correlating them with lipid inclusions of different appearance. The purified fraction was sonicated, treated with 1% Triton X-100 for 20 minutes on ice, mixed with the same volume of 80% sucrose, applied at the bottom of 5 and 30 % step sucrose gradient and spun for 22 hours at 100,000xg. The detergent resistant fraction was collected, an aliquot fixed for electron microscopy before extraction in a methanol/chloroform/water system. The chloroform phase was analyzed resulting in 70.3% cholesteryl ester, 6.9% cholesterol and 22.4% phospholipids. Morphologically, the fraction corresponds to planar bilayers found inside intact reservosomes. Moreover, the

presence of GM1 ganglioside detected with cholera toxin B subunit in the reservosome detergent resistant membrane fraction was an unexpected result. Different lipid domains in reservosomes may reflect specialized storing modes or *en bloc* lipid targeting for degradation.

2791

**Plasma Membrane ABCG1 Mediates Cellular Cholesterol Efflux to Lipoproteins**

E. B. Neufeld, K. O'Brien, S. J. Demosky, J. A. Stonik, S. L. Sabol, D. Malide, C. A. Combs, A. T. Remaley, S. Santamaina-Fojo, H. B. Brewer; NHLBI, NIH, Bethesda, MD

The ABCG1 transporter has been shown to play a role in the removal of cellular cholesterol by HDL and other lipoprotein acceptors. However, to date, little is known of the intracellular localization or site(s) of function of ABCG1. In the present study, we show that the carboxy-terminal GFP-tagged human ABCG1 transporter stably expressed in HeLa cells resides on the cell surface and traffics from the cell surface to cholesterol-enriched late endocytic vesicles and back to the cell surface. ABCG1-GFP on the cell surface (i) increases both plasma membrane and intracellular cholesterol, and, (ii) generates plasma membrane detergent-resistant cholesterol domains within which it does not itself reside. ABCG1-GFP expressing cells abnormally retain exogenously supplied fluorescent sphingomyelin on the cell surface and in endosomes. ABCG1-GFP expression enhanced efflux of cellular cholesterol to lipoproteins. ABCG1, unlike the ABCA1 transporter, supported neither the uptake nor lipidation of apoA-I, or, efflux of cellular phospholipids to lipoproteins. Taken together, these studies suggest that ABCG1 on the cell surface and endosomes generates pools of cholesterol that transfer specifically to extracellular lipid-laden lipoproteins. These studies establish that the ABCG1 and ABCA1 transporters generate different types of membrane cholesterol pools that are available for removal from the cell by distinctive mechanisms, involving lipoproteins (diffusion), and, lipid-poor apoA-I (micosolubilization), respectively.

2792

**Endocytic Processing in Living HuH7 and Trf1 Cells by Microscopy Assay: Defect in Vesicle Maturation in Trf1 Cells**

S. Nath,[1] J. W. Murray,[1] R. J. Stockert,[2] A. W. Wolkoff[3]; [1]Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]Liver Research Center, Albert Einstein College of Medicine, Bronx, NY, [3]Anatomy and Structural Biology and Liver Research Center, Albert Einstein College of Medicine, Bronx, NY

The Trf1 cell line was selected from mutagenized human hepatoma (HuH7) cells following dual selection schemes using two toxic ligands that are endocytosed by distinct receptors. Trf1 cells are defective in cell surface receptor trafficking, characterized by intracellular trapping of receptors related to absence of a novel casein kinase 2 isoform, CK2α". As little is known regarding ligand trafficking in these cells, the present study was designed to investigate the trafficking of an endocytosed ligand asialoorosomucoid (ASOR), in HuH7 and Trf1 cells. **Methods:** Cells were cultured on MatTek dishes. In a typical single wave experiment the cells were incubated with Texas-Red labeled ASOR (10µg) for 30min at 4°C, washed and warmed up at 37°C. Digital images were captured at 1frame per second and analyzed using ImageJ software. **Results:** At early times (2min to 12min) following 37°C incubation, fluorescent ASOR was seen in intracellular vesicles that moved and distributed throughout the cell. After 12min of incubation, vesicles in HuH7 cells were seem to fuse, resulting in substantially increased fluorescence content (~5 times) and apparent size of individual vesicles. This fusion did not occur in endocytic vesicles within Trf1 cells, even after 60min following 37°C incubation. **Conclusion:** Although both cell types ultimately deliver endocytosed ligand to lysosomes, these data indicate marked differences in trafficking and maturation of endocytic vesicles between HuH7 and Trf1 cells. The mechanism resulting in reduced vesicle fusion in these endocytic trafficking mutants remains to be elucidated.

2793

**Subcellular Nanomanipulation**

M. Nolan,[1] G. Hughes,[1] R. Gupta,[1] C. Mikoryak,[2] R. K. Draper[2]; [1]Zyvex Corporation, Richardson, TX, [2]Molecular and Cell Biology, University of Texas at Dallas, Richardson, TX

The dimensional scales of nanotechnology and cell biology meet at the subcellular level. One consequence of this convergence is that new tools developed for nanoscale applications are available for the study of cells. Described here is work with a nanomanipulator that has four actuators, each capable of controlling the movement of attached probes in three dimensions with a precision of 5 nm. The instrument is mounted on an inverted stage microscope with differential interference contrast (DIC), wide field, and confocal fluorescence capability. Tungsten probes whose tips were coated with platinum for interaction with thiols were functionalized with quantum dots so that the fluorescent tips of the probes could be followed with high-resolution fluorescence microscopy. The objective of this work was to determine whether the probes could be inserted into living cells and influence specific subcellular structures. The first model system explored was to generate sucrosomes in normal rat kidney (NRK) cells by incubating the cells with 30 mM sucrose, followed by staining the sucrosomes with an acidotropic fluorescent dye for visualization by fluorescence microscopy. Individual sucrosomes were then targeted for nanoprobing. We show that individual sucrosomes can be touched, nudged, pierced and affected by nanomanipulation within living cells. Future work will include nanomanipulation of other subcellular organelles.

2794

**Cellular Requirements for Human Rhinovirus 2 Uncoating**

U. Berka,[1] M. Brabec,[1] D. Blaas,[2] R. Fuchs[1]; [1]Department of Pathophysiology, Medical University of Vienna, Vienna, Austria, [2]Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria

Human rhinoviruses (HRV) are non-enveloped (+) RNA viruses. HRV2 - a minor receptor group virus - is internalized by lipoprotein receptors, dissociates from its receptors in early endosomes (pH $\leq$ 6.5) and undergoes uncoating at pH $\leq$ 5.6 in late endosomes. The RNA is translocated across the endosomal membrane into the cytoplasm where viral replication occurs. Uncoating can also take place at the plasma membrane when bound HRV2 is artificially exposed to pH $\leq$ 5.6 buffer. To analyze whether the pH gradient between endosomal or extracellular milieu and the pH-neutral cytoplasm is the driving force for RNA translocation we used membrane permeable (acetate pH 5.0) and impermeable [4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) pH 5.0] buffers to induce virus uncoating at the plasma membrane of HeLa cells. The membrane permeability versus impermeability of these buffers was verified by determining the intracellular pH with a pH-sensitive dye. Two hours incubation in EPPS buffer did not affect the cytoplasmic pH whereas the cytoplasm rapidly equilibrated with the extracellular pH upon incubation

in acetate buffer. HRV2 was bound to HeLa cells (4°C, pH 7.4) followed by low pH treatment with EPPS or acetate (1 h, 4°C) to induce uncoating. Cells were then transferred into growth medium and the number of cells synthesizing viral protein was determined by immunofluorescence microscopy after 17 hrs. Incubation with membrane-permeable buffer (pH equilibrium conditions) slightly stimulated HRV2 infection and thus RNA membrane translocation as compared to membrane-impermeable buffer (conditions that maintain the pH gradient). This demonstrates that a pH gradient as present during the normal (endosomal) infection route is not required for RNA transport into the cytoplasm. Supported by Austrian Science Fund P17590.

2795

**Tracking NGF Signaling Endosomes with Quantum Dots: One at a Time**

C. Wu,[1] B. Cui,[2] A. Ramirez,[1] K. Zhan,[1] L. Chen,[2] W. C. Mobley,[1] S. Chu[2,3]; [1]Neurology and Neurological Sciences, Stanford University Med Ctr, Stanford, CA, [2]Physics and Applied Physics, Stanford University, Stanford, CA, [3]Lawrence Berkeley National Laboratory, Berkeley, CA

Nerve growth factor (NGF) is a target-derived neurotrophic factor that plays a critical role in regulating neuronal survival and differentiation. It is well established that NGF binds to and activates its receptor, TrkA, at axonal terminals to elicit trophic signaling cascades in the corresponding cell bodies. A long-standing question is how the NGF signal is transported over substantial distances without losing the ability to induce robust and highly specific responses. The signaling endosome hypothesis addresses this issue. It stipulates that NGF binds to and activates TrkA at the axonal surface to recruit an array of signaling molecules from cytosol. Endocytosis of the NGF/TrkA/signaling proteins gives rise to signaling endosomes. The signaling endosome engages the microtubule network and dynein to effect retrograde transport to the cell body where it influences gene expression and activates other responses. In recent years strong evidence has been produced to support the hypothesis. However, direct visualization of the NGF signaling endosome during its genesis, activation and axonal trafficking has been lacking due to technical constraints. In the present study, we used biotinylated NGF in conjunction with a streptavidin-quantum dot 605 conjugate (BtNGF-Str-QD605) to track the real time movement of NGF in compartmentalized DRG cultures. NGF-containing endosomes traveled within the axon in a-stop-and-go fashion with an average speed of 1.6 μm/s. Furthermore, more than 99% moved only retrogradely. Importantly, BtNGF-Str-QD605 displayed significant colocalization with Trk, pErk1/2, and Rab5, all of which are integral components of the NGF signaling endosome. In a separate study, we demonstrated that inhibition of retrograde transport of NGF signaling endosomes caused atrophy in mature and death of immature DRG neurons. Taken together, these findings support the existence of NGF signaling endosome and point to a critical role in neuronal survival and maintenance.

## Leukocytes (2796-2807)

2796

**Modeling and Quantifying Leukemia Cell Deformability with Force Microscopy**

M. J. Rosenbluth,[1] W. A. Lam,[1,2] D. A. Fletcher[1]; [1]Department of Bioengineering, UC Berkeley, Berkeley, CA, [2]Department of Pediatrics, Division of Hematology/Oncology, UC San Francisco, San Francisco, CA

Atomic force microscopy (AFM) has become an important tool for quantifying mechanical properties of biological materials ranging from single molecules to cells and tissues. Current AFM techniques for measuring elastic and viscoelastic properties of cells are based on indentation of cells firmly adhered to a substrate, but these techniques are not appropriate for probing non-adherent cells, such as passive human leukocytes, due to a lateral instability of the cells under load. Here we present a method for characterizing non-adherent cells with AFM by mechanically immobilizing them in microfabricated wells. We apply this technique to compare the deformability of human myeloid and lymphoid leukemia cells at low deformation rates, and we find that both cell lines are well described by an elastic model based on Hertzian mechanics rather than a liquid droplet model frequently used to describe normal leukocytes. Myeloid leukemia (HL60) cells were measured to be a factor of 18 stiffer than lymphoid leukemia (Jurkat) cells on average ($E$ = 855 ± 670 Pa for HL60 cells, $E$ = 48 ± 35 Pa for Jurkat cells, mean ± SD). This work demonstrates a simple method for extending AFM mechanical property measurements to non-adherent cells and can be used as an assay to determine the effects of cytokines, chemotherapeutics, and cytoskeleton-perturbing agents on leukocyte deformability.

2797

**Sirpβ1 Is Expressed as a Disulfide Linked Homodimer in Leukocytes and Positively Regulates Neutrophil Transepithelial Migration**

Y. Liu,[1] I. Soto,[1] Q. Tong,[1] K. Zen,[2] A. Chin[2]; [1]Biology, Georgia Sate University, Atlanta, GA, [2]Pathology, Emory University, Atlanta, GA

Signal regulatory proteins (SIRPs) comprise a family of cell surface signaling receptors differentially expressed in leukocytes and the central nervous system. Although the extracellular domains of SIRPs are highly similar, classical motifs in the cytoplasmic or transmembrane domains distinguish them as either activating (β) or inhibitory (α) isoforms. In human neutrophils (PMN), previously studies from our group have demonstrated multiple SIRP isoforms and that SIRPα binding to its ligand CD47 regulates PMN transmigration. However, given the highly homologous extracellular domain structures of SIRP family members, it was difficult to rigorously define identity and functional role of other SIRP proteins in PMN. In the present study, we utilized SIRPα and β isoform-specific antibodies and RT-PCR to define the major SIRP isoforms in human PMN. We identified that Bit/PTPNS-1 and SIRPβ1 are the major SIRPα and SIRPβ isoforms expressed in PMN, respectively. Furthermore, we found that, while SIRPα (Bit/PTPNS-1) is expressed as a monomer, SIRPβ1 is expressed on the cell surface as a disulfide-linked homodimer with bond formation mediated by Cys320 in the membrane proximal Ig loop. Subcellular localization by sucrose gradient fractionation studies revealed a major pool of SIRPβ1 within plasma membrane of PMN. In contrast, the majority of SIRPα (Bit/PTPNS-1) is present in fractions enriched in secondary granules and is translocated to the plasma membrane after chemoattractant (fMLP) stimulation. Functional studies revealed that antibody-mediated ligation of SIRPβ1 enhanced fMLP-driven PMN transepithelial migration. Co-immunoprecipitation experiments to identify associated adaptor proteins revealed a 10-12 kDa protein associated with SIRPβ1 that was tyrosine phosphorylated after PMN stimulation and is not DAP10/12 or FcRγ. These results provide new insights into the structure and function of SIRPs in leukocytes and their potential role(s) in fine-tuning responses to inflammatory stimuli.

2798

**Fas Activation Reduces Neutrophil Chemotaxis in Response to IL-8 but not fMLP**

B. Hendey, B. Desai, S. Lidder; Pharmacology, Rush University Medical Centre, Chicago, IL

Background: The loss of effector functions, including chemotaxis, has been observed in apoptotic Polymorphonuclear neutrophils (PMN) and may serve to limit inflammation. The expression of phosphatidylserine (PS) on the cell surface is important because they target PMN for removal by macrophages and are thought to play a role in limiting the inflammatory response. Objective: It has not been determined if the reduction in chemotaxis precedes PS externalization or if it is merely a consequence of cell death. Methods: We induced apoptosis in freshly isolated PMN by activating the Fas receptor using either soluble Fas ligand or a Fas activating antibody and examined both the timing of PS externalization and changes in chemotaxis. Results: An increase in PS externalization was not observed until after three hours of Fas activation, but there was no effect on subsequent fMLP-stimulated chemotaxis. Further, fMLP treatment reduces PS externalization in the Fas treated cells from 31.45 ±2.43% to 22.91 ± 3.96 %. In contrast to the results with fMLP, there was a reduction in IL-8-stimulated chemotaxis that preceded PS externalization. Flow cytometry indicates that treatment with either Fas activating antibody or sFasL for one hour reduced the mean fluorescence of the IL-8 receptors, CXCR1 and CXCR2 by about 20%. Fas activation also reduced IL-8-induced Akt phosphorylation. Conclusions: These results indicate that early effects of Fas activation on chemotaxis are agonist specific. Since IL-8 is classified as an intermediate chemoattractant and bacterial peptides such as fMLP are classified as a dominant or end target chemoattractant, the results are discussed within the context of FasL reducing the continued recruitment of neutrophils by an intermediate chemoattractant, IL-8, without affecting the migration of PMN close enough to the site of infection to encounter bacterial peptides.

2799

**Structural Characterization of Sea Urchin Colorless Spherule Coelomocytes: Uniquely Motile Cells that Resemble Neutrophils**

M. B. Schott, T. J. Givens, A. Sturycz, L. Winslow; Biology, Northwestern College, St. Paul, MN

Colorless spherule coelomocytes are immune cells in the sea urchin, *Lytechinus variegatus*. These cells are ~30µ diameter, contain intracellular granules and possess a single flagellum. Digital video microscopy of live cells revealed a highly unique motility where the whip-like motion of the flagellum cause cells to spin in a circular or semi-circular motion with little or no directional translocation. Our approach was to use vital stain fluorescence imaging to determine the spatial intracellular distribution of organelles as a means of cellular characterization. DAPI staining revealed a large, single, nucleus that often appeared dumbbell shaped. The lobed nucleus was routinely found in the upper left quadrant of the cell opposite the point of attachment of the cell's single flagellum. DiOC6 staining revealed a bright lacework of endoplasmic reticulum that appeared to exclude the nuclear region. The fluorescent dye R123 showed even distribution of mitochondria throughout the cell. Colorless spherule cells stained with NBDC6, a fluorescent golgi marker, revealed accumulated clusters of fluorescence patches restricted to the same quadrant as the nucleus. Using DIC optics, numerous colorless granules are easily observed within the cytoplasm. These granules do not appear to be motile within the cell and are all of approximately the same size and dimension. Staining with the acidic dye, acridine orange revealed numerous fluorescent granules that seem to correlate with granules in companion DIC images. Colorless spherule cells were separated on Percoll gradients and intracellular granules were isolated by differential centrifugation. Isolated granules were analyzed by fluorescence staining with DiOC6 and acridine orange. Isolated granules stained brightly with both DiOC6 and acridine orange. Taken together, these data indicate that the colorless spherule coelomocytes many be akin to granulocytes in higher vertebrates such as neutrophils.

2800

**Filopodia Formation in the KG1a Human Hematopoietic Cell Line and Earthworm Coelomocytes under Hypertonic Osmotic Stress**

M. D. Dutt, M. R. Kasschau, S. K. Murphy; Biological Sciences, University of the Sciences in Philadelphia, Philadelphia, PA

The human acute myelogenous leukemic cell line, KG1a extrudes long thin pseudopodia or "osmopodia" in medium with increasing salt concentrations (Oh et al, 2000). Similar podia, known as filopodia have been observed to form in earthworm (*Lumbricus terrestris*) coelomocytes (leukocytes) under high osmotic conditions (Ngo and Kasschau, 2004). Time course experiments performed indicate that when isotonic IMDM (Iscove's Modified Dulbeccos Medium) 255 mOsm is increased to 745 mOsm almost 30% of KG1a cell population shows "osmopodia" within 15 mins and more than 90% show "osmopodia" at the end of 1 hour of exposure to the hyperosmotic medium. KG1a cells exposed to 575 mOsm and 635 mOsm media show numerous, short podia whereas cells in higher osmolality media (695 mOsm and 745 mOsm) show few, long podia. Similar to the earthworm coelomocytes, exposure of KG1a cells to the actin depolymerizing agent, cytochalasin D, enhances the formation of "osmopodia" even when the cells are maintained at 255 mOsm (4% to 48% increase in "osmopodia" forming KG1a cells) and also does not inhibit the formation of "osmopodia" at 745 mOsm. Cytoskeletal studies on the earthworm filopodia suggest that they are based on microfilaments and microtubules (Ngo and Kasschau, 2004). Similar morphological responses to hyperosmotic conditions are exhibited by leukocytes from two diverse species. We hope to determine whether there is a common model of leukocyte adaptation to osmotic stress. In both cases podia probably help these mobile cells in the search for more suitable environmental conditions.

2801

**The Role of Focal Adhesion Kinase (FAK) during the Innate Immune Response: Implications for Macrophage Migration, Cytokine Production and Phagocytosis**

K. A. Owen, B. Rey, A. H. Bouton; Microbiology, University of Virginia, Charlottesville, VA

The Role of Focal Adhesion Kinase (FAK) during the Innate Immune Response: Implications for Macrophage Migration, Cytokine Production and Phagocytosis As an important component of the innate immune system, macrophages have the ability to infiltrate sites of infection, initiate inflammatory responses and eliminate invading pathogens. In the present study, the use of mice in which the non-receptor tyrosine kinase FAK has been conditionally knocked-out in cells of macrophage lineage has allowed us to investigate the role of this molecule in common macrophage functions. Preliminary data indicate that FAK-deficient macrophages isolated from these mice exhibit altered spreading morphology by failing to produce abundant lamellipodia and filopodia 24 hours after plating on fibronectin. These cells also appear to respond with slower migratory kinetics compared to their wildtype littermates during wound healing assays. Furthermore, production of the pro-inflammatory cytokine IL-8 was strongly reduced in FAK-null macrophages upon infection with the gram-negative enteropathogen *Yersinia pseudotuberculosis*, a response that

may be due to an inability to efficiently activate ERK1/2. Finally, in some instances, phagocytosis of *Y. pseudotuberculosis* by these FAK-null macrophages was impaired. Taken together, these data suggest that FAK may constitute a vital link between multiple cell-surface receptors such as integrins, which are involved in migration and adhesion, and toll-like receptors (TLRs), which are involved in the recognition of pathogen-associated molecular patterns. Future experiments will help elucidate how this multifunctional molecule contributes to unique signaling outcomes in response to infection.

2802

**Iron Modulation of Protein Tyrosine Phosphatase Activity in Macrophages**

M. A. Gomez,[1,2] M. Olivier[1,2]; [1]Microbiology and Immunology, McGill University, Montreal, PQ, Canada, [2]Centre for the Study of Host Resistance, Montreal, PQ, Canada

Protein tyrosine phosphorylation is a fundamental determinant in the outcome of a wide array of cellular processes, ranging from cell proliferation to cell death. Regulatory mechanisms of protein tyrosine phosphatases (PTP) in the cellular context are not fully understood. However, events including tyrosine phosphorylation, protein dimerization and oxidation have been proposed as biological modulators of PTP activity. Here we present iron as a modulator of PTP activity in a macrophage model. Inhibition of PTP activity by ferric citrate in macrophage protein lysates is comparable to that of the specific PTP inhibitor $Na_3VO_4$. Iron-dependent PTP inhibition is a reversible event: rescue of activity in iron treated protein lysates upon addition of the iron chelator desferoxamine (DFO) is evident. This inhibition is not a consequence of the iron-dependent generation of reactive oxygen species as determined by superoxide dismutase (SOD) and catalase treatment. Moreover, *in-silico* analyses indicate putative iron binding sites in the catalytic pocket or in its near vicinity in more than 20 human PTPs. In relation to macrophage functions, our results indicate that iron-loaded macrophages have a reduced level of PTP activation upon *Leishmania* infection, together with a differential chemokine gene expression profile. All together, our data shows a novel mechanism of PTP activity modulation by one of the most important and biologically relevant transition metals, iron. These findings open new and interesting avenues in the research of cellular iron metabolism and protein tyrosine phosphorylation homeostasis. Our ongoing research aims to answer questions regarding the molecular mechanisms underlying iron-dependent PTP activity modulation, and its putative implications in multiple cellular functions.

2803

**Activation of Primary Macrophages after Efficient Gene Transfer**

C. Thiel, K. Hein, A. Krumnow, M. Weigel, T. Males, N. Faust; Research and Development, amaxa GmbH, Koeln, Germany

The response of macrophages and monocytes to environmental stimuli, like for example hypoxia-induced chemokine expression, LPS-induced cytokine production, or activation through bacterial DNA, plays an important role in immunity and inflammation. The molecular dissection of the underlying signal transduction cascades has mostly had to rely on macrophage cell lines that are relatively easy to transfect. Corresponding studies in primary cells have been hampered by the fact that the only way to obtain genetically modified primary macrophages or monocytes was through viral infection or by isolating them from transgenic animals. It has thus also been very difficult to examine the effects of intracellular delivery of plasmid DNA containing CpG motifs on the activation status of primary macrophages; a question that is clearly important for gene therapy approaches involving myeloid cells. In the study presented here, we show efficient gene transfer into primary human and murine macrophages as well as human mononcytes by the non-viral nucleofection® method. Using a GFP-reporter construct we routinely obtain between 45 and 60 % transfection efficiency while the cells mainly retain viablity and function. We also show that primary macrophages, like macrophage cell lines, respond to the uptake of plasmid DNA by tumor necrosis factor alpha (TNFα)-production. TNFα-secretion ceases after several hours and the cells can subsequently be re-stimulated by bacterial lipopolysaccharide. These findings now for the first time make it possible to analyse in detail the function of signal transduction proteins in normal non-infected primary macrophages or monocytes.

2804

**Activated Macrophages Express and Secrete Collagen Type VI**

M. Schnoor, P. Cullen, B. Brennhausen, K. Stolle, J. Rauterberg, S. Lorkowski; Leibniz-Institute for Arteriosclerosis Research, Muenster, Germany

In response to inflammatory signals blood monocytes migrate into affected tissues and differentiate to mature macrophages in order to remove the cause of the inflammation. Macrophages synthesize proteases and are therefore thought to have destabilizing effects on the extracellular matrix, which in the case of the arterial wall is synthesized mostly by smooth muscle cells. In previous work, we showed that - contrary to expectations - macrophages produce collagen type VIII *in vitro* and in the atherosclerotic plaque, indicating that under certain conditions these cells may contribute to plaque stability and the structural integrity of tissues in general. Following from this result, we screened human monocyte-derived macrophages *in vitro* for the expression of other collagens using RT-PCR. Surprisingly, we found that both monocytes and macrophages express almost all collagen mRNAs. We selected collagen type VI for further study because it anchors cells to the extracellular matrix and may therefore contribute to tissue stability. On Western-Blot analysis, collagen type VI was found in the medium of freshly isolated monocytes, but appeared in increasing amounts during differentiation to mature macrophages. Inhibition of intracellular transport or glycosylation by monensin or tunicamycin reduced the amount of this collagen secreted by macrophages in cell culture, but secretion was not inhibited by nocodazol, indicating that tubular structures are not involved in this process. Secretion was increased by TGF-β and interleukins 4 and 10, which induce alternative activation of macrophages. We also found that the function of collagen type VI in macrophages *in vitro* appears to be participation in β1-integrin-mediated cell adhesion. Overall, our results show that macrophages have both synthetic and degrading properties due to their ability to express both proteases and collagens. This suggests a general role for these cells in tissue remodeling.

2805

**Activation of $A_{2A}$ Adenosine Receptors Regulates Chemokine-stimulated Integrin-mediated Adhesion and Cell Spreading of CD4+ T Lymphocytes under Shear Flow**

R. K. Polanowska-Grabowska,[1] J. A. DiVietro,[2] J. Linden,[3] M. B. Lawrence[2]; [1]Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, [2]Biomedical Engineering, University of Virginia, Charlottesville, VA, [3]Division of Cardiovascular Medicine, University of

Virginia, Charlottesville, VA
Integrin-dependent adhesion, actin polymerization and cell spreading in T lymphocytes can be rapidly induced by chemokines such as SDF-1 (CXCL12). VLA-4 (α4β1) and LFA-1 (αLβ2) are the major integrins expressed on circulating lymphocytes which bind to ligands VCAM-1 and ICAM-1, respectively, on inflamed endothelium. Adenosine is an endogenous-purine nucleoside formed as the result of the breakdown of adenine nucleotides that are released from ischemic tissues, activated platelets, mast cells, and dying cells. In- vitro, $A_{2A}$ adenosine receptor agonists act, at least in part, through cyclic adenosine monophosphate (cAMP)-mediated pathways. Although $A_{2A}$ receptors possess anti-inflammatory properties their involvement in chemokine-stimulated lymphocyte adhesion under shear flow has not been studied. We have investigated if the specific adenosine receptor agonist ATL313 modulates integrin-dependent adhesion in SDF-1-stimulated human CD4+ lymphocytes. Adhesion assays to immobilized ICAM-1, VCAM-1 and P-selectin were performed using a parallel plate flow chamber at wall shear stresses of 0.5 to 1.0 dyne/cm$^2$. Treatment of CD4+ lymphocytes with ATL313 (1 μM) blocked SDF-1-stimulated adhesion to immobilized ICAM-1 and low density VCAM-1 (250 sites/μm$^2$) up to 80 and 50%, respectively. ATL313 also completely blocked cell spreading on ICAM-1. A combination of a suboptimal dose of ATL-313 (10 nM) with the type IV phosphodiesterase inhibitor rolipram (100 nM) synergistically decreased SDF-1-stimulated adhesion to ICAM-1 and to low density VCAM-1. The cAMP-dependent protein kinase A inhibitor (14-22 amide, cell-permeable, myristoylated) not only reversed the blocking effect of ATL313 but also stimulated adhesion and cell spreading on ICAM-1. Increasing cAMP with forskolin or loading with the cell-permeable cAMP analog blocked chemokine-stimulated adhesion and spreading. Therefore, activation of $A_{2A}$ adenosine receptors may counteract chemokine-stimulated integrin activation and adhesion to inflamed endothelium via a cAMP/PKA-mediated pathway.

2806

### Intercellular Transfer of Cell Surface Proteins across Immunological Synapses

G. S. Williams, E. N. Nolte-'t Hoen, D. M. Davis; Division of Cell and Molecular Biology, Imperial College London, London, United Kingdom
It is now well established that cell surface proteins and membrane components transfer between immune cells. Several possible mechanisms for this may be envisaged: membrane fusion, proteolytic cleavage, uprooting of transmembrane proteins or transfer of exosomes, cell fragments or membrane. We have previously observed bi-directional transfer of full-length proteins across NK cell immunological synapses, therefore refuting proteolytic cleavage as a possible mechanism. Using transwell assays, we now show that intercellular protein transfer is contact-dependent and that the cytoplasmic dye calcein does not transfer. Thus, exosomes or other membrane compartments containing cytoplasm do not mediate transfer. Transfer of proteins is greatly increased when the opposing cell possesses a cognate binding partner, and for example, the extent of transfer of the NK cell receptor KIR2DL1 strongly correlates with the level of expression of its ligand, HLA-C, on target cells. However we observe transfer of multiple proteins including GPI-linked GFP, for which there cannot be a physiological receptor, and thus to some extent, proteins can transfer independently from specific recognition. Unexpectedly, we also found that expression of HLA-Cw6 in NK cells causes a reduction in acquisition of HLA-Cw6 from target cells. This could reflect endogenous MHC protein occupying KIR2DL1 on the NK cell surface due to a *cis* interaction, analogous to that recently demonstrated for murine Ly49 NK cell receptors. Thus in summary, we demonstrate that upon contact, NK and target cells exchange fragments of membrane enriched in proteins for which there are binding partners on the opposing cell. Transferred proteins constitute a lasting molecular imprint of NK-target cell interactions, which we speculate may modulate subsequent cell behaviour.

2807

### Effect of the PI3-kinase/PTEN Pathway on PKC Phosphorylation in Jurkat T lymphocytes

M. Freeley,[1] Y. Volkov,[2] J. Park,[3] R. Wange,[4] D. Kelleher,[2] A. Long[1]; [1]Biochemistry, Royal College of Surgeons in Ireland, Dublin, Ireland, [2]Clinical Medicine, Trinity College, Dublin, Ireland, [3]Pharmacology, Chungnam National University, Taejon, Republic of Korea, [4]Gerontology Research Centre, National Institute of Health, Baltimore, MD
The Jurkat leukemic T cell line is deficient in the expression of the lipid phosphatase PTEN, the loss of which results in unopposed PI3-kinase activity and therefore high basal levels of 3'-phosphoinositides such as PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$. As a consequence of this deficiency, kinases that are dependent on 3'-phosphoinositides such as PKB/Akt are constitutively activated and phosphorylated (including the activation-loop residue Thr308). The PKC family of kinases also possess a conserved activation-loop residue analogous to Thr308 of PKB/Akt, and similar to PKB/Akt, this site is phosphorylated by the 3'-phosphoinositide-dependent protein kinase-1 (PDK-1). In this study we have addressed whether unopposed PI3-kinase activity in Jurkat T cells also affects PKC phosphorylation at the activation-loop site. We demonstrate that reducing the level of 3'-phosphoinositides in Jurkat cells with pharmacological inhibitors of PI3-kinase or expression of PTEN reduces PKB/Akt phosphorylation at Thr308 but has no effect on the corresponding activation-loop phosphorylation of PKC isoforms. We also demonstrate that the dephosphorylation of PKB/Akt at Thr308 is dependent on the activity of PP1/PP2a phosphatases and is not a consequence of reduced PDK-1 activity. We conclude therefore that the PI3-kinase/PTEN pathway does not affect PKC activation-loop phosphorylation in T cells.

## Cell Culture (2808-2825)

2808

### Purification and Characterization of Laminin-5 from Conditioned Media of Cultured Human Foreskin Fibroblasts

S. O'Malley, K. Kingsley; Biomedical Sciences, University of Nevada, Las Vegas School of Dental Medicine, Las Vegas, NV
Laminins are extracellular proteins primarily present at the basement membrane, where they provide structural stability and exert many biological functions, including cell adhesion, migration, proliferation and differentiation, and may also be involved in angiogenesis and tumor invasion. We screened several human cell lines obtained from American Type Culture Collection for the expression of human laminin-5 subunits (alpha3, beta3, and gamma2) using monoclonal antibodies specific for the gamma2 subunit (Chemicon mAb. 19562, mAb. 1947, mAb. 1949). Monoclonal antibodies for the gamma2 subunit were utilized for affinity purification as the alpha3 subunit is not exclusive to laminin-5, but rather is found in other laminin isoforms, such as laminin-6 and laminin-7. One cell line, Human Foreskin Fibroblast (hFF) cell line CRL-1634 Hs27, obtained from American Type Culture Collection, expressed measurable amounts of laminin-5 into the cultured media one day post confluence, which was recovered after centrifugation and subsequent fractionation. These results are being verified by RT-PCR and Western analysis. These results suggest one possible, alternative source and method of laminin-5 purification from the conditioned media of this cell line.

2809

**Ceramide Induces Morphological Changes and Necrosis in Tumour Cells from Human Cervix-uterine Cancer: Possible Molecules Involved**

G. Gutiérrez Iglesias,[1,2] A. Parra Barrera,[1] E. Reyes Maldonado,[3] R. López-Marure[2]; [1]Urology, Hospital Infantil de Mexico, Mexico D.F., Mexico, [2]Physiology, National Institute of Cardiology, Mexico D.F., Mexico, [3]Morphology, Instituto Politecnico Nacional, Mexico D.F., Mexico

Ceramide is a potentially important mediator of several agents that affect cell growth, viability, and apoptotic and no apoptotic death. This lipid is derived from sphingomyelin hydrolysis, or produced by the de novo synthesis. We previously have demonstrated that ceramide inhibits the proliferation and induces death like necrosis of cervix-uterine cancer cells (CaCu) at a concentration of 3 μM, but it does not inhibit the proliferation of normal cells, although the mechanism for that is unknown. In this work, we evaluated the antiproliferative effect of ceramide C-6, the morphological changes and possible molecules that might be involved in the ceramide-induced death in three lines from CaCu. Morphological changes were evaluated with Papanicolaou staining, NFκB translocation was performed by electrophoretic mobility shift assay, reactive oxygen species (ROS) was determined by flow cytometry, and glutation production was quantified by HPLC. Ceramide C-6 induced morphological changes in cytoplasm and nuclei like necrosis. The translocation of NFκB and the production of ROS were increased by ceramide C-6. Finally, low levels of glutation were detected in the cells treated with ceramide C-6. These results indicate that ROS, NFkB and glutation are involved in ceramide C-6-induced necrotic death.

2810

**Experiences of EBV Transformation for Human Blood Cell Biobank**

I. Chang,[1] J. Wu,[2] H. Lu,[1] H. Ko,[1] J. Kuo,[1] C. Wang,[1] S. Hwang[1]; [1]Bioresource Collection and Research Cneter, Food Industry Research and Development Institute, Hsinchu, Taiwan Republic of China, [2]Institute of Biomedical Sciences, Taipei, Taiwan Republic of China

The cell laboratory of Bioresource Collection and Research Center (BCRC) in Food Industrial Research and Development Institute joined to establish the Taiwan Han Chinese Cell and DNA bank that was initiated by the Institute of Biomedical Sciences, Academia Sinica, Taiwan in 2003. This cell-based genetic bank will be a medical platform for exploring in genetic disease research especially more frequent occurrence in Chinese. In this project, blood samples collected all over Taiwan were sent to BCRC for B cell immortalization. Briefly, mononuclear cells are isolated from blood samples by ficoll density gradient centrifugation and incubated with Epstein-Barr virus for 1 hour. After remove of Epstein-Barr virus, the infected lymphocytes are incubated at 37°C, 5% $CO_2$ atmosphere. The transformed cell lines will be successfully established during 8-10 weeks growth. After stringent quality control, including mycoplasma and microbial contamination detection, the cells will be stored in liquid nitrogen tank with computerized data management. The host genomic information will be preserved in these transformed cells forever. To date, more than 4000 human EBV-transformed cell lines have been established and preserved. The success rate for cell line establishment was 94%. In addition to this, we found that several factors including transport time of blood sample, density and viability of isolated mononuclear cells would have influence on transformation time required by B cell. A systematic data-retrieving intranet and barcode system were also established.

2811

**Cyclic Mechanical Strain Inhibits the Proliferation of Human Dermal Fibroblasts**

W. Durante, D. Ensenat, K. J. Peyton; Medicine, Baylor College of Medicine, Houston, TX

Fibroblasts play a critical role in wound healing by depositing extracellular matrix needed to restore structure and function to the injured tissue. Recent studies suggest that mechanical stress may enhance wound healing by stimulating the production of extracellular matrix from fibroblasts. In the present study, we investigated whether cyclic mechanical strain also influences the growth of fibroblasts. Cultured human dermal fibroblasts were plated onto pronectin or collagen-coated Bioflex plates and subjected to a physiologically relevant level of cyclic strain (6% at 1 hertz) using the Flexercell 3000 Strain Unit. Treatment of fibroblasts with fetal bovine serum (10%) under static conditions for 7 days stimulated a time-dependent increase in cell number. However, the application of cyclic strain markedly reduced the growth rate of fibroblasts. A significant decrease in the number of fibroblasts was first noted after 2 days and this became more pronounced after 7 days of cyclic strain. The cyclic strain-mediated decrease in fibroblast growth was observed in both pronectin- and collagen-coated plates, and was not due to cell detachment. Cyclic strain inhibited serum-stimulated DNA synthesis. In addition, flow cytometric experiments revealed that cyclic strain decreased the fraction of cells in S phase and increased the percentage of cells in the $G_0/G_1$ phase of the cell cycle. In conclusion, the present study demonstrates that cyclic mechanical strain (6% at 1 hertz) inhibits fibroblast proliferation by arresting them in the $G_0/G_1$ phase of the cell cycle. The ability of cyclic strain to inhibit fibroblast proliferation while stimulating collagen synthesis may possibly affect wound healing by focusing cellular resources and activity on matrix deposition.

2812

**Characterization of a Three-dimensional Culture of Cardiomyocytes: From the Cell to the Microtissue**

L. R. Garzoni,[1] M. D. Rossi,[2] V. P. Guarani,[2] A. D. N. Barros,[2] C. M. Takiya,[2] A. R. Nogueira,[1] D. Adesse,[1] M. N. L. Meirelles,[1] R. Borojevic[2]; [1]Department of Ultra-structure and Cell Biology-IOC, Fiocruz, Rio de Janeiro, Brazil, [2]Department of Histology and Embryology, ICB, UFRJ, Rio de Janeiro, Brazil

Strong differences on cells`s behaviour has been reported when comparing monolayer (2D) and threedimensional (3D) cell culture systems. In a 3D microenvironment the cells shows many aspects observed on tissue *in vivo* as for example the gene-function, extracellular matrix, growth factor production, responses to drug treatment and others. Here we presented a functional 3D cardiac microtissue from mono-dispersed expanded cell culture *in vitro*. Mouse embryos cardiomyocytes obtained from primary culture using trypsin and collagenase were maintained in DMEM with CFS and $CaCl_2$ at 37° C in 5% of $CO_2$ atmosphere. Utilizing a gravity-enforced system with a non adherent substrate, the isolated cells re-aggregated as spheroids and assembled in microtissues after 4 days. There was a direct correlation between the number of cells (3,000-25,000 cells) and the microtissue`s size (150-400μm). Most of microtissues produced with 3,000-6,000 cells were able to contract for more than 45 days and a time elapsed reduction on diameter and on the contractile activity was observed on microtissues with 25,000 cells. After ten days of culture, the immunofluorescence analyses showed extracellular matrix compounds as fibronectin, laminin and collagen IV. Most of cells consisted of cardiomyocytes, once they were positive for tropomyosin and conexin 43. The ultrastructural analyses showed cells presenting high number of

mitochondrias near of myofibrils, sarcoplasmic reticulum and intercalar discs. Other cellular types as fibroblasts were observed on the microtissues. Our results demonstrate that is possible to produce a functional cardiac microtissues *in vitro*, presenting cells highly differentiated, extracellular matrix and a long term contractile activity. We believe that it can be a powerful skill for studies on distinct fields of the basic and applied cardiovascular research including bioengineering, cellular migration analyses, parasites-host microtissues interactions, chemotherapy, angiogenesis, inflammation and cellular therapy.

2813

**Glycosylation of Tyrosinase is Necessary for It to Enter the MHC Class I Processing and Presentation Pathway**

M. Ostankovitch,[1] M. Van Lith-Altrich,[1] V. Robilla,[1] D. N. Hebert,[2] V. H. Engelhard[1]; [1]University of Virginia, Charlottesville, VA, [2]Departement of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA

Tyrosinase derived peptides bound to Major Histocompatibility Complex (MHC) class I molecules are recognized at the surface of melanomas by T lymphocytes. It has previously been proposed that processing and presentation of a $Tyr_{369}$ antigenic peptide containing a Asp in place of Asn at position 371 involves translation of tyrosinase in the endoplasmic reticulum (ER), export of full-length glycosylated tyrosinase to the cytosol, deglycosylation and deamidation in the cytosol by peptide N-glycanase (PNGase), degradation by the proteasome and retransport of deamidated peptides into the ER for association with MHC class I. The present work sought to provide greater insight into the identity of tyrosinase degradation intermediates and the role of glycosylation in protein degradation and antigen presentation. In melanoma cells treated with epoxomicin, proteasome substrates derived from tyrosinase were found as deglycosylated but also as unglycosylated forms in the membrane and in the cytosol. Glycosylated intermediates also were identified, but only when both proteasomes and PNGase activity were blocked. These results demonstrated that in addition to deglycosylated tyrosinase, unglycosylated and misfolded glycosylated tyrosinase molecules are also proteasome substrates. Little or no Asn-containing $Tyr_{369}$ epitope was presented on cells expressing either wild type or mutated unglycosylated tyrosinase, demonstrating the necessity of glycosylation for appropriate processing. Conversely, inhibition of PNGase blocked presentation of Asp-containing $Tyr_{369}$, demonstrating that deglycosylation was necessary for either deamidation or appropriate degradation. However, we found that inhibition of PNGase had no effect on epitope presentation in cells expressing an N371D tyrosinase mutant. These results demonstrate that glycosylation sites outside of the epitope are required for appropriate degradation of tyrosinase leading to epitope presentation.

2814

**A Short-term *in vitro* Assay for Anti-tumor Promoters Using Human Lymphoblastoid Cells Latently Infected Epstein-barr Virus**

H. Tokuda,[1] T. Konoshima,[2] M. Takasaki,[2] J. Takayasu,[1] H. Nishino,[1] A. Iida[3]; [1]Department of Molecular Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Chiba Institute of Technology, Chiba, Japan, [3]Department of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

The idea neoplastic development consists of at least two qualitatively different stages has gained general acceptance. The second, promotion stage has been suggested to be more critical in the development of human cancer that the first, initiation stage. Most tumor promoters do not bind DNA and are negative in Ames mutagenicity assay, whereas initiating carcinogenesis usually meet one or both these criteria. Recently, TPA only those other phorbol esters with tumor promoting activity and fatty acid, n-butyric acid have been found to induced the viral cyclin latently infected Epstein-Barr virus(EBV) genome carrying cells. We have suggested that a system consisting of EBV nonproducer Raji cells and each concentration of anti-tumor promoters, chemopreventive agents might be useful as a practical screening method of certain type of chemopreventive agents, anti-tumor promoters in our eatable and herbal plants. Interestingly, EBV-early antigen(EBV-EA) activation induced by TPA, can be diminished by the additional of chemopreventive agents, Brazilian herbal medicine, food colorants and vitamins to adequate culture medium. In this studies, we tried to arrange for the useful method of screening and detection of chemopreventive agents.

2815

**Assessment of Swine Sperm Quality after *in Vitro* Mycotoxin Zearalenone and Its Derivatives Exposure**

E. Benzoni,[1] F. Minervini,[2] F. Fornelli,[2] A. Giannoccaro,[2] M. Faustini,[1] R. Grande,[1] C. Mossali,[1] E. Munari,[1] M. Torre,[3] S. Villani,[3] V. Russo,[1] D. Vigo[1]; [1]Department of Veterinary Science and Technologies for Food Safety, University of Milan, Faculty of Veterinary Medicine, Milan, Italy, [2]Institute of Sciences of Food Production (ISPA), CNR, Bari, Italy, [3]Department of Chemical Pharmacology, University of Pavia, Pavia, Italy

Several chemicals out of different classes are capable of activating or blocking hormone receptor, in particular estrogen or androgen receptor. Particularly xenoestrogens (natural and synthetic products) can be present in foods and could induce modifications of reproductive functions in human and animals. Zearalenone (ZEA) and its derivatives (α and β-zearalenol) are fungal metabolites produced by *Fusarium* species frequent contaminants in cereals. For their known estrogenic activity in farm animals, we investigated the swine semen quality after *in vitro* exposure to ZEA and its derivatives (α and β-ZEA) by the assessment of viability (Trypan blue dye exclusion), motility (CASA), stability of chromatin structure (SCSA) and DNA fragmentation (Apo-BrdU). After 24h, all mycotoxins, at concentrations ranging from $10^{-6}$ to $10^{-4}$µM, modified the semen quality with different effects. ZEA induced DNA double-strand breaks (54%; $p< 0.05$), evaluated by flow cytometry, α-ZEA modified the sperm chromatin structure (64%; $p<0.001$), and β-ZEA provoked modification of kinetic parameters. Influences on viability, evaluated by membrane damage, were induced by higher concentrations ($10^{-2}$ to 1 µM) of mycotoxins. After 48 h of incubation, β-ZEA showed apoptosis induction (more than 50%) and increased instability of chromatin (14%) at low levels ($10^{-8}$- $10^{-6}$ µM). This is the first report on toxicity induced by ZEA and its derivatives in swine spermatozoa. Exposure to ZEA and its derivatives could influence sperm quality, considering the widespread occurrence of mycotoxins in cereals and the low concentrations found to be active in this study.

2816

**Reproductive Efficiency of Encapsulated Boar Semen for Artificial Insemination**

D. Vigo,[1] A. Riccardi,[1] S. Scocca,[1] M. Faustini,[1] S. Villani,[2] U. Conte,[3] V. Russo,[1] G. Ricevuti,[3] L. Sesso,[1] E. Benzoni,[1] M. Torre[3]; [1]Department of Veterinary Science and Technologies for Food Safety, University of Milan, Faculty of Veterinary Medicine, Milan, Italy, [2]Dipartimento di Scienze Sanitarie Applicate e Psicocomportamentali, Università di Pavia, Pavia, Italy, [3]Dipartimento di Chimica Farmaceutica, Università di Pavia, Pavia, Italy

Barium alginate controlled release capsules containing swine semen were developed to improve the preservation timespan of the spermatic cells and maximize the efficiency of a single artificial insemination procedure. Aim of this work was to evaluate the efficiency of this new technique in a swine herd: 2929 sows were artificially inseminated with a conventional dose (60.8% double/triple inseminations, control), or a single artificial insemination with controlled release capsules (39.2%) during the period autumn 2003/autumn 2004 (winter,15.7%; spring, 19.7%; summer, 20.7%; autumn, 43.8%). A multiple logistic regression model was applied, using pregnancy diagnosis and parturition rate in positively diagnosed sows as outcome variable. As independent variables, treatment, season and treatment x season interaction were considered. Pregnancy probability increase of 25% following capsule treatment with respect to conventional insemination (Odds Ratio =1.25, 95%CI=0.87-1.81, P=n.s.). Parturition probability increases of 69% (Odds Ratio =1.69, 95%CI=1.00-2.88, P<0.05) after capsule insemination with respect to control sows. Therefore, a single artificial insemination with controlled release capsules allows the same fecundity yields and higher parturition frequencies when compared to conventional artificial insemination procedures.

2817

**Screening Method of Short Term *in vitro* Assay For Nitric Oxide Scavenger Reagents**

H. Tokuda,[1] T. Akihisa,[2] R. Tanaka,[3] J. Takayasu,[1] F. Enjo,[1] H. Nishino[1]; [1]Department of Molecular Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]College of Science, Nihon Universty, Tokyo, Japan, [3]Department of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences, Osaka, Japan

The important physiological roles of nitric oxide(NO) suggest that donation of NO may be useful for the treatment of several states, and bioactivity of NO was indistinguishable from that of endothelium derived relaxing factor. Some pharmacological NO donors, that they may serve as natural strange and transport forms for bioregulatory NO and currently in use for the biological generation of NO. Other important feature of NO is a mutagenic compounds that can cause mutations in bacteria as well as chromosomal aberrations in rat primary lung cells and it is best known as a toxic reactive free radicals. NO is emerging as an important mediator of cytotoxicity and/or mutagenicity. In order to develop a possible *in vitro* screening model of scavenger against NO, we begin to explore the potential role of treatment scavenging of malignant NO production. Chang liver cells (human derived) from in DMEM were cultured for 3 days before treatment. NOR1(NO donor, (+-) -(E)-methyl-2-[(E) -hydroxyimin]-5-nitro-6-methoxy-3-hexenamide ) was added into culture dish and incubated for 1 h under $CO_2$ incubator as control. For screening assay, test samples to culture dish were observed under light-microscopy (x 100) without stained. All observed cells count for more than 250. The inhibitory ratio was then calculated for arrangement of data. Anti-oxidative reagents tested showed moderate inhibitory effects on NOR1 activation. In present paper, we designed a short-term *in vitro* assay for detecting NO scavengers, and test is simple to perform, reproducible and should be applicable for mass-screeni g of useful substances in our environment.

2818

**Cell Culture on Nanopillar Sheet - Analysis of Hela Cells Cultured on Nanopillar Sheet**

S. Nomura,[1] R. Nagano,[1] H. Kojima,[1] Y. Ohyabu,[1] K. Kuwabara,[2] A. Miyauchi,[2] T. Uemura[1]; [1]Nanotechnology Research Institute, AIST(Natl Inst. Adv. Industr. Sci. Technol.), Tsukuba, Ibaraki, Japan, [2]Materials Research Laboratory, Hitachi Ltd., Hitachi, Japan

We have succeeded in forming high-aspect-ratio structures (nanopillars) by nanoimprinting.. In this paper, the application of the nanopillar sheet as a new type of cell culture dish is introduced. Polystyrene films with the thickness of 1.0 μm were spin-coated onto glass substrates. By pressing the nano-mold made of silicon wafer onto the films at 150 $^{o}$Cand releasing the nano-mold from the films at room temperature, nanopillar structures were formed. The diameters of the nanopillars ranged between 0.16 μm and 1.0 μm according to the patterns on the nano-molds. The height of nanopillars was 1.0 μm. HeLa cells were seeded at $1.0x10^5$ cells/$cm^2$ on the nanopillar sheet and cultured for 5 days in DMEM containing 10% FBS at 37$^{o}$C in a humidified atmosphere of 95% air with 5% $CO_2$ . Analysis were performed by cell counting, morphological observation using a phase contrast microscope and immuno-histochemical observation of F-actin and vinculin on the cells using rhodamine-phalloidin and anti vinculin monoclonal antibody, respectively. The cells cultured on nanopillar sheets were not spread, but adhered only on the head of the pillars, keeping their original round shape. They grew and proliferated on the pillars. Since the adhered area of the cells to materials was small, the cells were easily removed from the sheet and collected just only by pipetting without conventional trypsinization method. The analysis of the HeLa cells cultured on the nanopillar sheets suggested that the nanopillar sheets are able to provide a new type of cell culture dish. A damage free subculture by enzymes is possible and the cells on the sheets proliferated in the same way as on the flat dishes with different manner of adhesion to the materials.

2819

**The Effect of Modified Media on the Expression of α-SMA and ALDH3A1 in Human Corneal Stromal Cells**

S. Rodenberg,[1] A. Tan,[1] A. Voorhees,[1] M. K. Bechtel,[1] E. J. Orwin[2]; [1]Biology, Harvey Mudd College, Claremont, CA, [2]Biology and Engineering, Harvey Mudd College, Claremont, CA

Normal human corneal stroma is composed primarily of quiescent keratocytes, characterized by expression of two water soluble proteins, aldehyde dehydrogenase 3A1 (ALDH3A1) and transketolase (TKT), which assist in corneal transparency. These cells are capable of differentiating into repair fibroblasts when injured or when grown in monolayer culture. Repair fibroblasts can be stimulated to reversibly differentiate into myofibroblasts (wound healing phenotype), characterized by increased α-smooth muscle actin (α-SMA) expression. When quiescent keratocytes become either of these cell types, there is a loss of transparency that may be attributed to the production of α-SMA, as well as the disruption of the matrix structure, resulting in an opaque cornea. The objective of this study was to determine how various culture conditions may affect the expression of ALDH3A1 and α-SMA in human corneal stromal cells. Western blot and immunofluorescence analysis demonstrated that media supplemented with transforming growth factor-β (TGF-β) increased α-SMA protein levels in these cells when cultured in monolayer. Quantitative real-time PCR analysis demonstrated that TGF-β increased α-SMA mRNA and decreased ALDH3A1 mRNA expression. Analysis via immunofluorescence confocal microscopy indicated that serum-free media supplemented with fibroblast growth factor (FGF) and heparin maintained α-SMA protein levels while decreasing ALDH3A1 protein levels. In addition, serum-free media alone was found to upregulate α-SMA and ALDH3A1 after seven days of treatment, while at eleven days α-SMA expression was found to return to normal. From these results we hypothesize that serum-free media supplemented with FGF and heparin induces differentiation of myofibroblasts to repair fibroblasts, and that serum-free media alone induces repair fibroblasts to revert to the quiescent keratocyte phenotype. Continuing studies will examine time

dependence of treatment with serum-free medium on α-SMA and ALDH3A1 protein and mRNA expression.

2820

**Continuous Cell Culture and Analysis in a Microfluidic Microbioreactor**

T. A. Holland,[1] E. Behravesh,[1] H. Sung,[1] K. Zygourakis,[2] S. R. Gonda[3]; [1]Division of Space Life Sciences, Universities Space and Research Association, Houston, TX, [2]Department of Chemical Engineering, Rice University, Houston, TX, [3]Advanced Biotechnology Section, NASA Johnson Space Center, Houston, TX

For many applications, it is desirable to sample cultured cells and medium over prolonged periods to examine the effects of a given factor on cell function. Many microbioreactor systems currently provide for continuous media sampling but, unfortunately, require experiment termination and reactor disassembly for cell sampling. The current work describes a microfluidic microbioreactor system developed in our laboratory, and a method for real-time cell sampling that eliminates the need for cell passaging or bioreactor disassembly. The microfluidic microbioreactor contains an annular designed chamber that is 3.30mm x 30.0mm x 0.25 mm (w x l x h), with multiple inlet and exit lines, and a transparent polycarbonate disk for light microscope monitoring. In this work, we investigated the effect of surface substratum and shear stress on L1210 cell adhesion by step-wise increases in flow rates at 10 min intervals to develop shear stresses of 0.13 to 0.45 dyne/cm2, followed by reversal of this trend with stepwise decreases at 10 min intervals. In addition, we examined the effect of alterations in shear stress on the size distribution of L1210 cells detached from the micro-culture chamber. Our results showed that low shear stresses (0.09-0.13 dyne/cm2) selectively detached daughter cells from a monolayer of exponentially dividing L1210 cells. Alternatively, higher shear stresses (0.22-0.45 dyne/cm2) were shown to detach a mixed population of both daughter and parent cells, while maintaining a monolayer of adherent cells in the micro-culture chamber. Accordingly, this microbioreactor design provides a cost and space efficient method of assessing the potential effects of numerous environment factors on cell behavior through controlled, real-time cell and media sampling. Supported by funding from NASA.

2821

**Mineralization Induced by Micro-Sized Pillars**

J. Justesen,[1,2] A. Fuechtbauer,[1] M. Duch,[1,2] M. Foss,[3,2] L. Andersen,[3,2] O. Hansen,[4] J. Chevallier,[3] F. Besenbacher,[3,2] F. Pedersen[1,2]; [1]Department of Molecular Biology, University of Aarhus, Aarhus C, Denmark, [2]Interdisciplinary Nanoscience Center, University of Aarhus, Aarhus C, Denmark, [3]Department of Physcics and Astronomy, University of Aarhus, Aarhus C, Denmark, [4]Department of Micro and Nanotechnology, Technical University of Denmark, Copenhagen, Denmark

Fast and stable biointegration of bone implants is important for the health and well being of patients after orthopaedic surgery. In order to identify promising surfaces, we have screened wafers with an array of multiple microstructures for mineralization properties. Silicon wafers with microstructures were coated with 250 nm tantalum. A total of 15 structures were designed on wafers with 5 different depths. MC3T3-E1 (murine preosteoblasts) were seeded onto the wafers in growth medium and stained for alkaline phosphatase after 1 and 2 weeks. Cells seeded in medium containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate were stained with Alizarin Red after 3½ weeks for mineralisation. For actin fibre visualisation, cells were grown for 48 hrs, fixed, and stained with phalloidine. Alizarin Red staining on wafers, visualised that mineralization was enhanced by the pillar-structure D1. Neither any other structure nor the slightly different pillar-structure D2 showed enhanced staining. Mineralization studies on the different depths illustrated a decrease in the staining intensity for D1, as compared to the other structures, with decreasing depth. In contrast, no difference between the microstructures was observed for the alkaline phosphatase staining. Elongated actin fibres was observed for cells grown on flat structures (reference) and D2. However, on the D1 structure, the actin fibres were deposited around the pillars and the shape of the cells were highly influenced by the pattern of the structure. In conclusion, the structure and depth of the growth surface affect the differentiation of the cells. MC3T3-E1 cells show increased mineralization on D1, a process affected by the depth of the structure. This increase in mineralization may be connected with the observed early changes in the cytoskeleton of the cells.

2823

**Establishing a Cell Biology Model of Suppression in Epithelial Ovarian Cancer**

D. Dafou,[1] D. A. Trott,[2] R. F. Newbold,[3] J. Timms,[4] S. J. Ramus,[1] I. J. Jacobs,[1] S. A. Gayther[1]; [1]Gynaecological Oncology, UCL, Institute of Women's Health, United Kingdom, [2]Transplant Immunology, Imperial College London, National Heart and Lung Institute, United Kingdom, [3]Life Sciences, Brunel University, Institute of Cancer Genetics and Pharmacogenomics, United Kingdom, [4]Biochemistry, UCL, Ludwig Institute, United Kingdom

Epithelial ovarian cancer is the leading cause of death from gynaecological malignancy worldwide. Identifying molecular markers of disease may provide novel approaches to screening and could enable targeted treatment and/or lead to the design of novel therapies. Unfortunately, the molecular genetic events that underlie the initiation and progression of ovarian cancer are poorly understood. Previously141 primary epithelial ovarian tumours were analysed using metaphase CGH analysis to identify common regions of chromosome deletions that may indicate the presence of tumour and/or metastasis suppressor genes. Complete or interstitial deletions of chromosomes 4, 5, 6, 13, 14, 15, 18 and 21 occurred in 36-68 % of all tumours analysed, suggesting that these chromosomes may harbour suppressor gene(s). Subsequently, we have used the microcell mediated chromosome transfer (MMCT) to introduce these chromosomes into EOC cell lines in order to assess the their functional significance. *In vitro* and *in vivo* data indicate that chromosomes 5, 6 and 18 have a strong negative effect on ovarian cancer cell growth and anchorage independent colony formation. This suggests these chromosomes contain one or more genes that behave as ovarian cancer tumour and/or metastasis suppressor genes. We have mapped candidate gene locations on chromosome 18 by microsatellite repeat analysis. We have analysed RNA expression array data and 2-Dimension Differential Gel Electrophoresis data from hybrids, parentals and revertant cell lines. In doing so, we aim to identify genes and biological pathways that represent new therapeutic targets for EOC treatment.

2824

**Novel Three-Dimensional Cell Scaffold Allows Non-enzymatic Cell Release**

E. C. Elliott,[1] S. L. Snabes,[2] J. Bang,[1] S. Wang,[3] N. Kotov[1]; [1]Chemical Engineering, University of Michigan, Ann Arbor, MI, [2]Research, Aastrom Biosciences, Ann Arbor, MI, [3]Nomadics Inc., Stillwater, OK

We recently reported a new type of three-dimensional scaffold with inverted-colloidal-crystal geometry. Here we introduce a positively charged super absorbent polymeric hydrogel matrix. The cationic hydrogel is based on a crosslinked copolymer network consisting of N, N-dimethylacrlyamide and (3-Acrylamidopropyl)trimethyl-ammonium chloride. The topography of the scaffold consists of uniform spherical cavities interconnected by a network of channels with openings ranging from 104 to 300 µm. Scanning electron and confocal microscopy have confirmed that the cationic nature of the matrix allows for remarkable cell adhesion and nesting throughout the hydrophilic three-dimensional matrix without using costly or time consuming coating techniques. Furthermore, by adjusting the media multivalent ion concentration, we are able to dynamically control and manipulate swelling equilibrium and the size of the interconnecting openings during cell incubation. With up to a 10-fold increase in swelling equilibrium we are able to reversibly expand and collapse the interconnected cavities from 100-600 µm in size. Such action provides a reagent or enzyme free method of cell release from our matrix. This engineered scaffold possesses desirable optical, functional, and mechanical properties that can also facilitate tissue regeneration, continuous high-resolution optical monitoring of cell proliferation and cell-cell interaction. Biocompatibility has been demonstrated with HS-5, MSC, and OP9 cell cultures. When cultured with various feeder layers, cells appear to attach and expand normally. Cytospins, cell and viability counts, confocal microscopy, SEM and flow cytometry confirm that morphology and function are preserved during culture and harvest. This functionality, ease of production, unique 3D structure, biocompatibility, optical transparency, and the non-enzymatic alternative for cell harvest combine to make this new type of hydrogel scaffold advantageous for today's demanding cell and tissue culture field.

2825

### Development of an Avian Expression System for Chicken 1,25$D_3$-MARRS

S. E. Safford; Biology, Lincoln University, Lincoln University, PA

1,25$D_3$-MARRS (Membrane Associated Rapid Response to Steroids)/ERp57 is a recently identified protein mediating rapid ion transport responses to vitamin D hormone, 1,25$D_3$, in chicken intestine. To acquire the full length avian protein for structural and functional analysis, we wished to express the protein in a recombinant system. To date, avian codon bias prevented efficient expression of the chicken protein in a variety of mammalian expression systems lacking essential tRNAs. To solve this problem, we transfected a plasmid encoding full length chicken 1,25$D_3$-MARRS linked to a myc-his tag at the C-terminus into a quail sarcoma (QT-6) cell line. The protein encoded by the transfected plasmid was recognized by a commercial anti-myc monoclonal antibody, and was slightly larger than endogenous 1,25$D_3$-MARRS expressed by the quail cells. Both proteins were recognized by an antibody raised against the N-terminus of chicken 1,25$D_3$-MARRS purified from basolateral membrane (Ab099). Rat intestinal epithelial cells (IEC-6 and IEC-18) transfected with the chicken 1,25$D_3$-MARRS plasmid failed to express a protein recognized by the anti-myc antibody. However, endogenous rat 1,25$D_3$-MARRS was recognized by a commercial mammalian antibody to the C-terminus of ERp57. Rat 1,25$D_3$-MARRS migrated slower in SDS-PAGE gels than did endogenous quail 1,25$D_3$-MARRS. We conclude that the quail model is superior for expression of the 1,25D3-MARRS protein for investigation of cell surface expression of 1,25$D_3$-MARRS, for studies of cell trafficking of 1,25$D_3$-MARRS under unstimulated and 1,25$D_3$ stimulated conditions, and for pharmacological study of binding of 1,25$D_3$ to the expressed 1,25$D_3$-MARRS protein.

## Cancer IV (2826-2846)

2826

### HMG-CoA Reductase is Required for the Initiation and Maintenance of MYC Induced Tumorigenesis

C. M. Shachaf,[1] O. D. Perez,[2] S. Youssef,[3] M. Chang,[1] S. V. Elchuri,[1] M. J. Goldstein,[3] L. Steinman,[3] G. P. Nolan,[2] D. W. Felsher[1]; [1]Medicine/Oncology, Stanford University, Stanford, CA, [2]Microbiology & Immunology, The Baxter Laboratory for Genetic Pharmacology, Stanford University, Stanford, CA, [3]Neurology and Neurological Sciences, Stanford University, Stanford, CA

The best therapeutic targets for cancer may not be the immediate molecular underpinnings of tumorigenesis, but key nodal proteins of metabolic signaling pathways. The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is a critical enzyme in the mevalonate pathway, required for the synthesis of several important bioactive compounds. We demonstrate in a defined model of oncogene induced tumorigenesis that the inhibition of HMG-CoA reductase was sufficient to not only reverse established tumors, but to prevent MYC induced tumor development. Even briefly treating adult transgenic mice with the inhibitor of HMG-CoA reductase, atorvastatin prevented tumor onset. Bone marrow from tumor affected animals could be purged by *in vitro* treatment with atorvastatin and used to reconstitute the hematopoietic system in lethally irradiated mice. To account for these findings, we defined the phospho-protein profiles associated with the inhibition of HMG-CoA reductase by treatment with atorvastatin. Atorvastatin inactivated the MYC oncogene through the inhibition of Ras and ERK1/2, resulting in the prevention of MYC phosphorylation and activation. Treatment with the down-stream metabolite of HMG-CoA reductase, mevalonate, prevented statins from reversing the phospho-protein signature and tumorigenesis associated with MYC. Also, RNAi directed at HMG-CoA reductase was alone sufficient to abrogate the neoplastic properties of MYC induced tumors. Thus, HMG-CoA reductase appears to be an example of a key regulatory protein required for both tumor initiation and maintenance of tumors induced through MYC overexpression.

2827

### Effect of MUC-1 Expression on EGFR Endocytosis and Degradation in Human Breast Cancer Cell Lines

R. M. El Bejjani; Molecular and Cellular Biology, The University of Arizona, Tucson, AZ

EGFR overexpression and amplification have been tied to tumor formation in the mammary gland. Overexpression of MUC1 in the breast induces the formation of tumors that are able to metastasize. The mechanism by which MUC1 causes the onset of tumor formation is unknown, but studies show that MUC1 can modulate EGFR signaling. Our preliminary data shows that MUC1 and EGFR interact in breast cancer cells but not in the normal polarized mammary epithelium. Data generated in BT20 human breast cancer cell lines shows that MUC1 and EGFR interact in these cells only when treated with the EGFR ligand, EGF. Upon ligand binding, EGFR is typically endocytosed, where it is either degraded or recycled. We show that MUC1 knockdown in BT20 cells induces faster degradation of ligand-bound EGFR compared to MUC1-expressing cells. Localization of endocytosed EGFR demonstrates that it localizes to a perinuclear compartment of unknown identity where internalized MUC1 resides. To determine whether MUC1 affects the degradation of surface EGFR or previously internalized EGFR, we performed biotinylation of surface

proteins. These experiments show that MUC1 affects the degradation of ligand-bound EGFR at the cell surface. Future experiments will examine the non-clathrin mediated endocytosis pathways in order to determine the nature of the observed perinuclear organelle. We will also investigate how MUC1 overexpression affects Cbl, Grb2 and CALM association with EGFR during endocytosis (key proteins in the endocytosis and degradation of EGFR). Finally, we will investigate the effect of MUC1 overexpression and knockdown on the tumorigenic potential of human breast cancer cell lines and in mouse models.

2828

**Effects of hTERT on Metal-induced Changes in Clonogenic Survival**

A. Glaviano,[1] D. M. Baird,[2] E. Cabuy,[3] M. A. Rubio,[4] P. Slijepcevic,[5] C. Mothersill,[6] F. Lyng,[6] C. P. Case[1]; [1]Dpt. of Orthopaedic Surgery, University of Bristol, Bristol, United Kingdom, [2]Department of Pathology, University of Cardiff, Cardiff, United Kingdom, [3]Brunel Institute of Cancer genetics and Pharmacogenomics, University of Brunel, London, United Kingdom, [4]Lawrence Berkeley National Laboratory, University of Berkeley, Berkeley, CA, [5]Brunel institute of Cancer Genetics and Pharmacogenomics, University of Brunel, London, United Kingdom, [6]Radiation and Environmental Science Centre, Dublin Institute of Technology, Dublin, Ireland

Genomic instability is considered to be an important component in carcinogenesis. Cr(VI) and V(V) can initiate long term genomic instability in generations of daughter cells from parent human primary fibroblasts exposed to metal ions for only 24 hours. This is regulated by telomerase. In wild type hTERT- cells there is a persistent increase of dicentric chromosomes. In hTERT+ cells (after ectopic transfection) there is a persistent increase in tetraploidy. In this study we have examined the clonogenic survival of these cells using the clonogenic assay. The telomere lengths were measured with telomere flow FISH and STELLA, the cell cycle with flow cytometry and the degree of senescence with B Galactosidase staining. Exposure to metals caused a persistent and dose related reduction in clonogenic survival in hTERT- cells up to 30 days post exposure. This was not seen in hTERT+ cells except at the highest doses. In contrast there was no persistent change in the cell cycle distributions of either hTERT- or hTERT+ cells after metal exposure if they were grown at non-confluent levels in flasks. Now was there a change in their proliferation rates. Only a short-term induction of apoptosis was seen. In the cells grown as colonies for the clonogenic assay there was a progressive and dose dependent increase in B galactosidase staining 30 days after metal exposure in hTERT- cells which was not seen in hTERT+ cells. A population of cells with short telomeres was present in hTERT- cells that was not seen in hTERT+ cells. However the distributions of telomere lengths were not altered by the metal exposures. This study suggests metals may alter the growth of human cells as colonies. Although this may be influenced by telomerase it may not be explained by metals changing telomere lengths.

2829

**A Possible Link Between Centrioles, Calcium Deficiency and Cancer**

J. Wells; Discovery Institute, Seattle, WA

Centrosomal defects leading to chromosomal instability appear to be an early step in the development of major human cancers, though the precise nature of the centrosomal defects remains unknown. An animal centrosome contains two orthogonally oriented centrioles, each composed of nine microtubule triplets arranged like the blades of a turbine. An engineering analysis of centriole ultrastructure based on electron microscope data leads to the hypothesis that these organelles function as dynein-driven turbines to generate a high-speed, small-amplitude oscillation in spindle microtubules that resembles the motion of a laboratory vortexer. Calculations show that the result would be a polar ejection force several times stronger than gravity that would tend to push chromosomes away from spindle poles during prometaphase and metaphase. [Rivista di Biologia / Biology Forum 98 (2005): 71-96]. The transient increase in intracellular calcium that normally accompanies the onset of anaphase would shut down the dynein-driven centriolar turbines to permit the unobstructed poleward movement of chromosomes. Under conditions of calcium deficiency or defective calcium regulation, however, centrioles could generate an increasing polar ejection force throughout anaphase that would break chromosome arms and produce chromosomal instability. Several experimental approaches are suggested to test the hypothesis, which if corroborated may contribute to a better understanding not only of cell division but also of cancer.

2830

**Characterizing *Helicobacter pylori's* Virulence Factor, CagA, Using a *Drosophila* Model**

C. M. Botham, K. J. Guillemin; Institute of Molecular Biology, Uninversity of Oregon, Eugene, OR

*Helicobacter pylori's* virulence factor CagA is delivered via a Type IV secretion system into host cells and subsequently alters cell signaling. Inside cells, CagA is tyrosine phosphorylated by host Src kinases and activates SRC homology 2-domain phosphatase (SHP-2), a key component of receptor tyrosine kinase (RTK) pathways. Several proteins known to interact with CagA are also involved in RTK signaling. The activation of SHP-2 by CagA is hypothesized to be similar to the normal activation of SHP-2 by Grb2-associated binder (Gab) proteins. We are using a *Drosophila* model to determine if CagA functions like a Gab protein mimic. *Drosophila* are ideal for these experiments because their RTK pathways are well characterized and homologous to mammalian RTK pathways. We have generated transgenic *Drosophila* and shown that CagA activates several RTK pathways. The sevenless (SEV) RTK pathway specifies the R7 photoreceptor in the eye and CagA expression in the eye causes extra R7 photoreceptors. This phenotype mimics the expression of constitutively activated corkscrew (CSW), the *Drosophila* homologue of SHP-2. Daughter of sevenless (DOS), the only *Drosophila* homologue of Gab, normally activates CSW in the SEV RTK pathway and we are using *csw* and *dos* mutants to determine whether CagA acts solely through CSW and whether CagA mimics Gab. CagA expression in the tracheal system causes extra tracheal branches similar to expression of constitutively activated CSW in the fibroblast growth factor receptor (FGFR) RTK pathway. Downstream of FGF, scaffolding adaptor protein, normally activates the FGFR RTK pathway and we are using mutants to determine whether CagA acts as a general activator of RTK pathways. Dissecting CagA's activation of *Drosophila* RTK pathways will lead to a better understanding of the mechanisms by which CagA perturbs cell signaling.

2831

**Downregulation of Naked2 in Colorectal Cancer**

W. Ding,[1] J. Smith,[1] C. Li,[1] R. J. Coffey[1,2]; [1]Vanderbilt University, Nashville, TN, [2]Veterans Affairs Medical Center, Nashville, TN

Genetic studies in Drosophila have established that activation of canonical Wnt signaling results in upregulation of Naked Cuticle that then binds

and inactivates Dishevelled, a positive regulator of Wnt signaling. Two mammalian Naked homologs, Naked1 and Naked2, have been identified. We recently reported that Naked2, but not Naked1, interacts with the cytoplasmic tail of pro-TGFα, and escorts pro-TGFα-containing exocytic vesicles to the basolateral surface of polarized epithelial cells where these vesicles dock and fuse in Naked2 myristoylation-dependent manner (PNAS 101: 5571-5576, 2004). The present studies were designed to address whether Naked 1 and 2 were regulated like Naked Cuticle. For these studies, we utilized parental SW480 cells, a human colorectal cancer (CRC) cell line with mutant APC and active Wnt signaling as determined by nuclear β-catenin, and clones stably transfected with APC in which β-catenin is redirected to the plasma membrane and the cells revert to a non-transformed phenotype. Like Naked Cuticle, Naked1 expression by RT-PCR is high in parental SW480 cells and decreased in APC-transfected SW480 cells. In contrast, Naked2 expression is barely detectable in parental SW480 cells and both mRNA by RT-PCR and protein by western blotting are increased in APC-transfected SW480 cells. Moreover, immunohistochemical staining for TGFα is redirected from the cytoplasm to the plasma membrane in APC-transfected SW480 cells. In addition, full length 55kDa Naked2 protein is downregulated in 5 of 6 paired normal/cancer human CRC specimens by western blotting. We hypothesize that Naked2 helps to maintain normal epithelial homeostasis by regulated basolateral cell surface delivery of TGFα, and that loss of Naked2 predisposes to neoplasia.

2832

**Regulation of the Proteasome by Yin6/int6 and an Actin Regulatory Protein, Arc21**

R. Cabrera, E. C. Chang; Breast Center, Baylor College of Medicine, Houston, TX

The “int” genes were originally identified as loci where mouse mammary tumor virus (MMTV) integration induces breast tumor formation. Nearly all “int” genes have well characterized molecular functions that are critical for oncogenesis; in contrast, the role of *int6* in tumorigenesis remains largely unknown. In order to better understand how *int6* can mediate cancer formation, we have recently uncovered a highly conserved function of *int6* by characterizing the *int6* ortholog, named *yin6*, in the genetic model system fission yeast. Our data suggest that *int6* regulates localization and assembly of the proteasome. Inactivation of *int6* weakens the proteasome leading to chromosome instability and abnormal mitosis. To further decipher how Int6 regulates proteasome localization and assembly, our lab performed a screen to isolate *S. pombe* cDNAs that, when overexpressed, rescue the growth defect of *yin6* null (*yin6*Δ) cells. This study focuses on *arc21*, encoding a potential subunit of the Arp2/3 complex, which has been implicated in the regulation of actin polymerization. My gene knock out experiments show that *arc21* is an essential gene and its inaction is associated with loss of cell polarity. Furthermore, my data show that an Arc21-GFP fusion protein colocalizes with F-actin, a pattern that can be disrupted by the actin polymerization inhibitor Latrunculin A. These results support the hypothesis that Arc21 binds actin and regulates actin function. Overexpression of Arc21 can rescue the proteasome defects observed in *yin6Δ* cells. Additionally, *yin6Δ* cells show an aberrant localization of Arc21-GFP, and, like many mutants defective in F-actin, are hypersensitive to actin polymerization inhibitors. These data support a model in which *yin6* mediates proteasome localization and assembly by acting via *arc21* and the actin cytoskeleton.

2833

**Erbb2 Disrupts Epithelial Cell Polarity by Inactivating the Par Complex**

V. Aranda,[1] T. Haire,[1] M. E. Nolan,[1] J. P. Fawcett,[2] C. D. Allred,[3] T. Pawson,[2] S. K. Muthuswamy[1]; [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, [2]Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institue, Mount Sinai Hospital, Toronto, ON, Canada, [3]Breast Center, Baylor College of Medicine, Houston, TX

Loss of tissue organization is a feature observed in all pre-cancerous epithelial lesions. Oncogenes such as v-Src, Ras, Myc and ErbB2 are known to disrupt epithelial cell architecture in cell culture and mouse models. Epithelial architecture is characterized by apical-basal asymmetry, referred to as cell polarity. During polarity establishment, a series of protein complexes, Scribble/Lgl/Dlg, Crumbs/PALS1/PATJ and the Par3/Par6/aPKC complex interact hierarchically to generate the organizational asymmetry. There is little information about the role of these polarity regulators in oncogenic signaling, although several studies are starting to identify them as potential tumor suppressors in mammals. The mechanisms by which oncogenes can interact with the cellular machinery involved in regulating cell architecture remain unknown. We have developed a system to model the early stages of oncogene-induced transformation by overexpressing ErbB2 in MDCK cells. Using an inducible receptor, we have characterized the time course of events that leads to the disruption of apical-basal polarity upon ErbB2 activation. This phenotype resembles the alterations observed in early cancerous lesions and has lead us to identify tight junctions as the earliest target of oncogene action. In order to characterize ErbB2-induced disruption of TJ, we focused on the Par3/Par6/aPKC complex, known to play critical roles during TJ formation. We found that activation of ErbB2 induces the dissociation of Par3 from Par6/aPKC, and formation of an ErbB2-Par6/aPKC complex. This induces a decrease in Par6-associated aPKC activity, in contrast with aPKC upregulation described in polarity formation and transformation of non-polarized cells. Moreover, overexpression of a kinase dead version of aPKC phenotypically cooperates with ErbB2. Thus we identify a new pathway for ErbB2 oncogenic signaling in polarized cells that involves inactivation of the Par complex and promotes disruption of epithelial architecture.

2834

**Caspase-mediated Proteolysis of Neurofibromin**

M. K. Kuechle,[1] M. M. Shinohara,[1] K. G. Stephens[2]; [1]Medicine/Dermatology, University of Washington, Seattle, WA, [2]Medicine and Laboratory Medicine, University of Washington, Seattle, WA

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder with diverse clinical features that results from mutations in the NF1 gene. Neurofibromin, the NF1 protein product, is a large (2818 amino acids), multidomain protein, that functions in part as a tumor suppressor through its Ras-GTPase activating domain. The Gap-related domain (GRD) of neurofibromin accounts for only 10% of this large protein. The presence of multiple splice variants and the existence of pathogenic missense mutations outside the GRD suggest functions other than those related to the Ras pathway. Recently, NF has been shown to be actively transported to the nucleus via the presence of a bipartite nuclear localization signal in an exon located at the distal c-terminus. Nuclear staining for NF has been reported in developing neurons and in differentiating keratinocytes. Furthermore, altered nuclear localization of neurofibromin has been reported in psoriasis, a disorder of epidermal differentiation. These observations suggest that neurofibromin species found in the nucleus function to influence differentiation and/or development in certain tissues. A database screen identified neurofibromin as a potential caspase substrate with two consensus sites. In this study, we demonstrate that neurofibromin is proteolytically cleaved during Fas-mediated apoptosis in BJAB cells, and is due to a caspase-6 like protease. Furthermore, the cleavage pattern is

compatible with the predicted caspase sites. These findings identify neurofibromin as a novel caspase target, and provide the first evidence that neurofibromin is an important contributor to cell death as well as cell survival. Based on these studies, we hypothesize that caspase-mediated cleavage of neurofibromin releases the nuclear localization domain, allowing transport to the nucleus, where it functions to signal downstream events of cellular differentiation.

2835

**Vav2 Modulates Cellular Invasion Through the Rac1 Signaling Pathway**

S. Y. Lai,[1] A. F. Ziober,[2] M. N. Lee,[2] E. M. Falls,[2] B. L. Ziober[2]; [1]Otolaryngology, University of Pittsburgh, Pittsburgh, PA, [2]Otorhinolaryngology, University of Pennsylvania, Philadelphia, PA

The Rho family of GTPases regulate cellular adhesion and motility. Guanine nucleotide exchange factors (GEFs) in turn regulate the activity of GTPases by promoting nucleotide exchange from GDP to GTP. We have determined that GTP-bound Rac1 is elevated in invasive oral squamous cell carcinoma (OSCC). Furthermore, expression of Vav2, a Rho family GTPase for Rac1, Cdc42 and RhoA was also up regulated in OSCC. Although Vav2 may regulate a number of cellular functions the specific signaling pathways remain incompletely defined in OSCC. Because of the critical role of cellular invasion in the progression and metastasis of OSCC, we investigated if Vav2 modulated Rac1 and Cdc42 activation and thus influenced cellular invasion in OSCC. Transient expression of Vav2 in UM-22B-SCC, a highly invasive OSCC cell line, resulted in an increase in GTP-bound Rac1, but not Cdc42. Stable expression of Vav2 in UM-22B-SCC and HaCaT cells resulted in an increase in GTP-bound Rac1, and to a lesser extent, GTP-bound Cdc42. Increased levels of GTP-bound Rac1 correlated with increased cellular invasion. Adenovirus-mediated expression of a dominant-negative Rac1 in the HaCaT-Vav2 cells and invasive OSCC cells resulted in an inhibition of cell invasion This effect appeared to be specific for Rac1, as a dominant-negative Cdc42 affected cellular invasion to a lesser extent. Thus, Vav2 appears to modulate cellular invasion through specific regulation of Rac1 activity in OSCC.

2836

**Identification of a Gene Expression Signature Associated with Oral Squamous Cell Carcinoma**

A. F. Ziober,[1] P. A. Gimotty,[2] J. L. Hunt,[3] K. R. Patel,[1] M. Feldman,[4] B. L. Ziober[1]; [1]Otorhinolaryngology, University of Pennsylvania, Philadelphia, PA, [2]Biostatistics and Epidemiology, University of Pennsylvania, Philadelphia, PA, [3]Pathology, University of Pittsburgh, Pittsburgh, PA, [4]Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Attempts to find biomarkers that distinguish OSCC lesions from normal oral epithelium have resulted in several candidate genes associated with OSCC tumor progression including p53, cyclin D1, and EGFR. However, to date, no single gene has shown sufficient diagnostic utility in OSCC. In an effort to identify a gene expression signature that may serve as biomarkers for OSCC we analyzed 12 non-cancerous and OSCC matched specimens from the University of Pittsburgh and the University of Pennsylvania using Affymetrix U133A GeneChip arrays. A *t* test of the normalized values for each gene with an adjusted p-value of $p < 0.001$ and the Benjamini-Hochberg false discovery correction yielded a list of 63 genes. Expression of selected genes was validated by real-time PCR and immunohistochemistry. Hierarchal clustering and Treeview imaging software were used to visualize the gene signature. Comparison of the paired non-cancerous tissue with the corresponding OSCC tumors identified several gene signatures including those associated the extracellular matrix, proliferation and signal transduction. Interestingly, cluster analysis identified primary OSCC samples that were lymph node positive and a gene signature for site specific expression in the oral cavity. Using Prediction Analysis of Microarrays (PAM) and the 12 non-cancerous and OSCC matched specimens, we identified a set of genes that, on cross-validation, were 83-88% accurate at classifying non-cancerous and OSCC specimens. This gene set was able to predict two independent non-cancerous and OSCC mismatched groups of 27 and 21 specimens with accuracies of 93% and 90%, respectively. These data provide evidence for gene expression-based biomarkers in OSCC.

2837

**Targeting TACE-Dependent Growth Factor Shedding in Breast Cancer**

P. A. Kenny, M. J. Bissell; Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

The ability to proliferate independently of signals from other cell types is a fundamental characteristic of tumor cells. Tumors resulting from inappropriate activation of the EGFR are common in multiple tissues and are, for the most part, refractory to current targeted therapies. Using a 3D culture model of human breast cancer progression, we have delineated a protease-dependent autocrine loop which provides an oncogenic stimulus to this pathway in the absence of proto-oncogene mutation. Inhibition of this protease, TACE/ADAM17, reverts the malignant phenotype by preventing mobilization of two crucial growth factors, Amphiregulin and TGFα. We show further that the efficacy of EGFR inhibitors is overcome by physiological levels of growth factors and that successful EGFR inhibition is dependent on reducing ligand bioavailability. Using existing patient outcome data, we demonstrate a strong correlation between TACE and TGFα expression in human breast cancers that is predictive of poor prognosis**.** These data implicate TACE as a therapeutically tractable enzyme, the inhibition of which effectively blocks EGFR signaling by preventing mobilization of ligands for this receptor. Furthermore, the data provide mechanistic insight into the insensitivity of EGFR-overexpressing tumors to anilinoquinazoline inhibitors and suggest that co-ordinate inhibition of TACE might augment the activity of EGFR inhibitors in a clinical setting.

2838

**The Neurotrophin Receptor p75 Mediates Glioma Invasion**

A. L. M. Johnston,[1] X. Lun,[1] M. Slovack,[1] B. L. Hempstead,[2] S. M. Robbins,[1] D. L. Senger,[1] P. A. Forsyth[1]; [1]University of Calgary, Calgary, AB, Canada, [2]Cornell University Medical College, New York, NY

The invasive nature of cancers in general and malignant gliomas in particular is a major clinical problem and renders tumors incurable by “local” therapies (e.g. surgery, radiotherapy). To better understand invasion in glioma, we used serial *in vivo* selection to isolate highly invasive glioma cells from a non-invasive human glioma cell line, U87. Gene expression profiles of highly invasive cells were compared to their non-invasive counterparts using DNA microarrays. We found that the p75 neurotrophin receptor was significantly upregulated in the invading glioma cells. p75 was functional and ligand binding increased both migration and invasion *in vitro.* Ectopic expression of p75 in the original U87 cell line conferred

an increased migratory and invasive ability *in vitro* and upon implantation into SCID mouse brains. Conversely, down-regulation of p75, in a human glioma cell line expressing high levels of p75, decreased the migration and invasion of these cells *in vitro*. These data suggest that p75 is present, functional, and involved in glioma migration and invasion. In addition, high levels of p75 were common in patient specimens from highly invasive glioblastomas multiforme. Finally, as a beginning to understanding the mechanism behind p75's invasive effects, we have demonstrated that these cells also express the p75 ligand BDNF and may be setting up an autocrine loop. Future experiments are aimed at further elucidating p75's mechanism of action in this system. In glioma cells, reports have hinted at roles for p75 in growth and apoptosis; however, data presented here supports a much different role for p75, that of mediating glioma cell invasion.

2839

**3D Morphologies of Breast Tumor Cell Lines Cluster by Gene Expression Patterns**

C. Myers; Lawrence Berkeley National Lab, Berkeley, CA

Previous studies from the Bissell laboratory have shown that non-malignant and malignant cells can be distinguished easily by their morphological architecture when cultured in a three dimensional, laminin-rich extracellular matrix gels(3DlrECM) but not when cultured on traditional tissue culture plastic (2D)(1). In addition we have shown that cellular response to signaling inhibitors and apoptotic agents differ in cells cultured in 2D vs 3D(2-4). We asked whether the 3D morphology could be used as a reasonable surrogate for gene expression profiles for a panel of breast cancer cell lines. The 16 cell lines examined so far fell into 6 distinct morphologies termed round, round mass, irregular mass, grape-like, grape-like stellate and invasive stellate. Hierarchical clustering of 3836 genes with statistically significant differential expression clustered into 2 major groups: Stellates in one, and round/ mass/ and grape-like in the other. The 3D cluster analysis is similar to the 2D cluster analysis. However, the 3D morphological assignments allowed for finer subcatorgorization in that representatives from each of the 6 morphologies clustered together. We are currently analyzing these expression profiles to identify common signaling themes and/or morphological regulators as well as performing studies correlating morphology and expression profiles with therapeutic response to Herceptin and other therapeutic agents. 1. O.W. Petersen OW et al., Proc Natl Acad Sci U S A 89, 9064-68 (1992). 2. F. Wang et al., Proc Natl Acad Sci U S A 95, 14821-6 (Dec 8, 1998). 3. V.M. Weaver et al., Can Cell 2, 205-16 (Sep, 2002). 4. M.J. Bissell et al., Curr Opin Cell Biol (6):753-62 (2003).

2840

**Asbestos Produces Aneuploidy by Preferential Binding to Cell-Cycle Regulatory Proteins**

R. A. MacCorkle, S. D. Slattery, D. R. Nash, B. R. Brinkley; Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX

Asbestos is lightweight and chemically durable construction material commonly used in the USA until the 1970's and still used throughout the world today. Asbestos has been formally linked to the development of pleural mesothelioma, other lung cancers and lung-damaging fibrosis. The latency period for asbestos-linked lung cancer is 30-45 years after exposure, thus the incidence of asbestos-linked cancers is currently peaking in the USA and on the rise in other parts of the world. Unfortunately, our incomplete understanding of the carcinogenic mechanisms of asbestos has limited our ability to effectively prevent and treat disease caused by asbestos exposure. Studies have shown that the two common types of asbestos, amphibole and serpentine, can both physically interfere with chromosome segregation in human lung cells. This interference has been proposed to be responsible for the generation of a genetically unstable aneuploid cell population which has been linked to cancer progression. However, some cells still become aneuploid when exposed to asbestos in the absence of this physical interference mechanism. We hypothesized that an additional biochemical mechanism, such as sequestration of critical cell-cycle regulatory proteins, may contribute to asbestos-induced aneuploidy. To test this hypothesis we exposed asbestos fibers to human lung cell extracts and identified proteins that bound to them. We found that asbestos fibers preferentially bound proteins with known functions in regulation of the cell cycle, cytoskeleton and mitotic apparatus. This protein binding was functionally important because pre-coating of fibers with microtubule-associated protein (MAP)/ Tubulin complexes, effectively blocked cellular protein binding and aneuploidy generation in human lung cells. These results show a novel biochemical mechanism of protein sequestration by asbestos fibers that accounts for both amphibole and serpentine asbestos-induced aneuploidy. These results also show that precoating asbestos fibers with specific protein reduces their genotoxicity.

2841

**Altered Trafficking and Signaling of EGFR in Iressa-Sensitive Human Cell Lines**

P. J. Jasper, M. Lazzara, G. Lu, D. Lauffenburger, P. Sorger; Biology, MIT, Cambridge, MA

The small-molecule tyrosine kinase inhibitor Iressa belongs to a class of therapeutics aimed at interrupting dysregulated signaling by ErbB receptors (e.g., EGFR/ErbB1, HER2/ErbB2) in cancer. Recently, a strong correlation was established between patients who are responsive to Iressa and the presence of specific EGFR mutations. The relationship between mutation and Iressa sensitivity is not simple however; cells with WT receptor can be highly sensitive and even in mutants, sensitivity is not associated with tighter binding to the drug. The biology of WT and mutant ErbB receptors represents an opportunity to elucidate factors in the ErbB signal transduction pathway whose alteration leads to cancer, and to determine the precise mechanisms of action of anti-EGFR therapeutics. To gain quantitative, mechanistic understanding of ErbB1 mutations leading to Iressa-responsiveness, we employ a systems approach to investigating receptor trafficking, intracellular signaling, and cell death response in a panel of cell lines with wild type and mutant forms of EGFR. We are using this information to train physicochemical models of EGFR signaling under normal growth conditions and in the presence of Iressa or other kinase inhibitors. Analysis of EGFR trafficking reveals a striking pattern of lowered rate of internalization in Iressa-sensitive lines, regardless of whether EGFR is wild type or mutant. By probing for the phosphorylation of key downstream kinases, we have demonstrated differences in the signaling patterns of sensitive and insensitive cell lines. These differences are consistent with differential receptor trafficking. Ongoing research is aimed at the use of mathematical models to explain sensitivity and resistance, and eventually to develop design principles for second generation therapies.

2842

**Folate Receptor Targeting of a Transcription Factor Decoy for Hypoxia Induced Factor (HIF) for Treatment of Renal Cell Carcinoma**

Y. Li, C. Parham, E. Shocron, P. Nhonthachit, L. Komuves, J. Wilken, T. Flaherty, M. Garcia, J. Braisted, L. Beviglia, L. Powell, L. McEvoy, S. Hudak; Research, Corgentech Inc., South San Francsico, CA

The transcription factor Hypoxia-inducible Factor (HIF) controls expression of multiple genes involved in cancer biology, including angiogenesis, tumor growth, survival and invasion. Inhibition of HIF activity has marked effects on tumor growth. We generated a double-stranded oligonucleotide HIF Decoy that specifically binds to and inhibits HIF binding to target gene promoters. In previous work, we demonstrated that HIF Decoy blocks target gene expression in human tumor lines and reduces tumor growth by HIF Decoy in multiple murine xenograft tumor models. Efficacy equivalent to Avastin was seen in several xenograft tumor models where only 50% of the HIF activity was blocked, suggesting that improved delivery to the tumor is key to increased efficacy. Folate receptor is a highly selective tumor marker that has been utilized to deliver a wide variety of therapeutic agents. We developed a folate conjugate of our HIF Decoy in an effort to improve delivery to tumor cells. The renal tumor cell line 786-0 lacks HIF-1α but constitutively expresses HIF-2α, which is known to stimulate a number of hypoxia-inducible genes. We have demonstrated both the constitutive expression of HIF-2α and the binding of nuclear HIF2α by HIF Decoy in 786-O cells, as well as expression of folate receptor by flow cytometry. Folate-HIF Decoy demonstrates high affinity, specific binding and blocking of HIF-1α and HIF-2α activity. Folate conjugated HIF decoy showed a significant improvement in cellular and nuclear uptake compared to naked decoy, suggesting the potential for improved efficacy *in vivo. In vivo* experiments utilizing the 786-O xenograft tumor model are underway.

2843

**HoxA9 Modulates the Tumor Phenotype by Regulating BRCA1 Expression**

P. M. Gilbert,[1] M. K. Gbegnon,[1] J. N. Lakins,[1] M. A. Unger,[2] B. L. Weber,[3] V. M. Weaver[1]; [1]IME/Pathology and Lab Medicine, UPENN, Philadelphia, PA, [2]Abramson Family Cancer Research Institute, UPENN, Philadelphia, PA, [3]Abramson Family Research Center/MERCK, UPENN, Philadelphia, PA

The tumor suppressor BRCA1 is known to play a critical role in familial and sporadic breast cancers. While genomic deletions and mutations in BRCA1 are known to underlie the loss of BRCA1 expression in familial breast cancers, less is known about what contributes to down-regulation and/or loss of function of this tumor suppressor in sporadic breast tumors. We previously identified HoxA9 as a gene whose expression is often lost during malignant transformation of the mammary gland. HoxA9 re expression in mammary epithelial tumor cells (METCs) resulted in decreased cell migration, proliferation, invasion, and anchorage independence *in vitro* as well as reduced tumor burden, growth, metastases, and viability *in vivo*. In addition, we found that HoxA9 re expression regulates adhesion through altering integrin expression and adhesion activity resulting in morphogenic reversion of tumors grown within a three-dimension reconstituted basement membrane (3D rBM). Subsequent studies showed HoxA9 directly binds to and activates the promoter of BRCA1. To determine which tumor modulator effects were dependent on HoxA9 mediated BRCA1 up regulation we utilized a full length BRCA1 expression construct in METCs and a dominant negative BRCA1 expression construct in METCs re-expressing exogenous HoxA9. Using soft agar and wound closure assays we showed BRCA1 is responsible for HoxA9 mediated effects on anchorage independence and migration. In addition, growth of these cells within a 3D rBM revealed that BRCA1 represses cell proliferation and promotes mammary morphogenesis by inducing apical polarity. *In vivo* studies demonstrated HoxA9 mediated effects on tumor metastases are exerted in part through up regulation of BRCA1. These data suggest that HoxA9 may function as a novel tumor modulator in mammary epithelial cells through regulation of tumor suppressors such as BRCA1, and by altering stromal-epithelial interactions. (Supp: DISS0402407, DODW81XWH-05-1-330, NCI CA078731 and DAMD17-01-1-0368).

2844

**Quantitative Analysis of Energy Metabolism Pathways in Breast Cancer Cells Using Native NAD(P)H**

R. P. Walvick, A. A. Heikal; Department of Bioengineering, Pennsylvania State University, University Park, PA

The concentration of intrinsic NAD(P)H in live cells or tissues is a sensitive reporter of mitochondrial function. Conventional biochemical techniques require lysing the cell in order to measure the concentration of any biomolecules. In contrast, fluorescence measurements provide a noninvasive approach for qualitative imaging of NAD(P)H in vivo. However, standard fluorescence microscopy does not take into account the sensitivity of the fluorescence dynamics of NAD(P)H to its cellular environment and the physiological conditions of live cells. Here we report on quantitative analysis of NAD(P)H as a reporter of the cell physiology using a combination of steady-state and time-resolved two-photon (2P) fluorescence imaging microscopy. Our preliminary results on breast cancer (Hs578T) and normal (Hs578Bst) cells, which are derived from the same patient, indicate quantitative differences between the intrinsic NAD(P)H in these two cell lines under 740-nm illumination. Of particular interest here is the exact concentration and the spatial distribution of native NAD(P)H to quantify the partitioning of the energy budget between oxidative phosphorylation and glycolysis in cancer cells. We have also used 2P-fluorescence polarization imaging microscopy to map out the dipole-moment orientation of native NAD(P)H in the heterogeneous environment of live cells. These results will ultimately help understanding of the underlying molecular differences in energy metabolic pathways in cancer cells and may lead to the development of a sensitive and intrinsic diagnostic probe.

2845

**nSMase2 is Modulated by Oxidative Stress and Induces Apoptosis in Lung Epithelial Cells**

M. Levy, S. S. Castillo, C. Wang, T. T. Goldkorn; Internal Medicine, UC Davis School of Medicine, Davis, CA

Our laboratory demonstrated that exposure of lung epithelial cells to oxidative stress via hydrogen peroxide (H2O2) directly activates an endogenous membrane neutral sphingomyelinase (nSMase) to induce ceramide accumulation followed by apoptosis. Additionally, we found that glutathione negatively regulated nSMase and inhibited apoptosis. Purification studies of nSMase from the lung (of monkey or pig) indicated that there is more than one isoform of nSMase present in the lung epithelium. Cloning approaches based on homology with bacterial SMases identified two mammalian enzymes, nSMase1 and nSMase2 as putative nSMases capable of hydrolyzing spingomyeline in-vitro. In order to determine the role of nSMase2 in lung epithelial cells we first demonstrated that nSMase2 is present and functional in lung epithelial cells. Then, using 3 overlapping sets of primers based on identical regions between human and mouse cDNA for nSMase2, we cloned nSMase2 from human A549 lung epithelial cells and from rhesus monkey lung tissue. We then transfected A549 cells with the cloned nSMase2. The over-expression of nSMase2 resulted in 16-fold increase in basal enzyme activity, which could be quenched by the addition of 10 mM glutathione. When 100 μM H2O2 was added to cells over-expressing nSMase2, an additional increase of 8-fold in enzyme activity was obtained. Both basal and inducible activation of nSMase2 were impaired by siRNA knockdown of nSMase2. In addition, H2O2-Induced apoptosis was examined before and after silencing. While

H2O2 enhanced early apoptosis 1.6-fold, silencing of nSMase2 canceled this induction. Our results suggest that the nSMase isozyme nSMase2 is modulated by oxidative stress and is a key player in lung epithelial cell death.

2846

**The Tetraspanin CD151 Regulates Rumor Cell Intravasation by Mediating Matrix Interactions During Migration and Invasion of the Tumor Stroma**

A. Zijlstra, J. Lewis, H. Stuhlman, J. P. Quigley; Cell Biology, The Scripps Research Institute, La Jolla, CA

In vivo, metastatic dissemination is a process involving multiple sequential rate-limiting steps which ultimately result in the colonization of secondary sites. Matrix interactions mediated by integrin complexes are thought to play a key role in the adhesion, migration and invasion of tumor cells at multiple steps in the metastatic cascade. The tetraspanin CD151 associates with the α3β1 integrin complex on the cell surface and is thought to regulate ligand interactions. Using a CD151 function blocking antibody, we have demonstrated that CD151 regulates migration of tumor cells on multiple ECM components, not only those bound by α3β1. As a consequence CD151 can regulate tumor cell motility in the complex matrix of tissue stroma. Cell surface CD151 clusters into micro-domains in response to antibody binding. The clustered CD151 exhibits improved association with the β1 integrin complexes and facilitates an increased formation of Paxillin containing adhesion complexes. Intravital imaging of tumor cell motility demonstrates that antibody ligation results in diminished migration due to the inability of the tumor cell to detach the trailing end of the cell body. Using high-throughput quantitative analysis of individual steps in the metastatic cascade, we have demonstrated that this failure to migrate does not affect primary tumor growth nor tumor cell arrest, extravasation or growth at the secondary site. In contrast, the inhibition of CD151 mediated migration prevents invasion of the tumor stroma and migration along the stromal vasculature. Quantitative assessment of intravasation demonstrates that the inhibition of CD151 mediated migration specifically prevents intravasation at the site of the primary tumor. Thus CD151 regulates migration in the stroma of tumor tissue. The inhibition of CD151 mediated migration prevents entry into the tumor vasculature and subsequent colonization of secondary tissues.

## Neuronal Diseases II (2847-2873)

2847

**Curing of Yeast [*PSI*$^+$] Prion by Guanidine Inactivation of Hsp104 Does Not Require Cell Division**

Y. Wu,[1] L. E. Greene,[1] D. C. Masison,[2] E. Eisenberg[1]; [1]Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, MD, [2]Laboratory of Biochemistry and Genetics, NIDDK, National Institutes of Health, Bethesda, MD

Propagation of the yeast prion [*PSI*$^+$], a self-replicating aggregated form of Sup35p requires Hsp104. One model to explain this phenomenon proposes that, in the absence of Hsp104, Sup35p aggregates enlarge but fail to replicate thus becoming diluted out as the yeast divide. To test this model, we used live imaging of Sup35p-GFP to follow the changes that occur in [*PSI*$^+$] cells after addition of guanidine to inactivate Hsp104. Following guanidine addition there was initially an increase in aggregation of Sup35p-GFP. But then, before the yeast divided, the aggregates began to dissolve, and after about 6 hours the Sup35-GFP looked identical to the Sup35-GFP in [*psi*$^-$] cells. Although plating studies showed that the yeast were still [*PSI*$^+$], this reduction in aggregation suggested that curing of [*PSI*$^+$] by inactivation of Hsp104 might be independent of cell division. This was tested by measuring the rate of curing of [*PSI*$^+$] cells in both dividing and non-dividing cells. Cell division was inhibited by either adding either alpha factor or farnesol. Remarkably, with both of these methods, we found that the rate of curing was not significantly affected by cell division. Thus cell division is not a determining factor for curing [*PSI*$^+$] by inactivating Hsp104 with guanidine. Rather, curing apparently occurs because Sup35-GFP polymers slowly depolymerize in the absence of Hsp104 activity. Hsp104 then counteracts this curing possibly by catalyzing formation of new polymers.

2848

**Prion Proteins [PrP] of Mammals, Yeast, Fungi and Others Are Related in Conserved Homologous Domains for Binding *Endogenous* Redox- and Metalloregulated *"Non-Coding"* Small RNA Bioaptamers: Codes for Epigenetic Reprogramming Cell Fate in Conformation Phase Pathway-Locked Loops [CPLL]**

J. H. Wissler, J. E. Wissler; ARCONS Institute for Applied Research & Didactics, Postfach 1327, D-61231 Bad Nauheim, Germany

OBJECTIVE: Relations of different PrP in interaction with entitled structurally defined *endogenous* nucleic acids to epigenetic [non-Mendelian] phenotype alterations [EPA] in reprogramming metabolic and developmental cell fate and proteinaceous transmission ["infection"] of conformational disease. METHODS: Isolation of such nucleic acids from wound and supernatant fluids of cells cultured in serum-free synthetic media. Purification guided by bioassays of cellular differentiation and tissue morphogenesis [Wissler et al., Protides Biol.Fluids 34:517-536,1986; Materialwiss.Werkstofftech.32:984-1008,2001; Ann.N.Y.Acad.Sci.991:333-338,2003; 1022:163-184,2004; FASEB J.19:A621,2005; FEBS J.272-s1:N1-06-4P,2005]. RESULTS: Bioactive *endogenous* complexes of nucleic acid bioaptamers and conformers of PrP-surrogate [S100-EF-hand] proteins were isolated. Some sequence-defined bioaptamers are redox- and metalloregulated, edited and modified *"non-coding"* small RNA [<200 bases] as regulators and messengers of protein structure [folding] at cellular translation and transcription levels. They address conserved homologous metalloregulated nucleic acid-binding domains in proteins, termed K/R3H-[K/RxxxH], i.e. -t/s/xK/R/q/nxxxH/y/n/q/e/dx$_{7-9}$h/xx$_{7-9}$h/xx$_{5-20}$K/R/q/n/e/h- with accessory basic [R/K]$_n$, R/K-zipper, SR/K/RS and/or HxxxH/y/n/q segments. Highly conserved homologous domains in mammalian PrP were found characteristically patterned in prions of yeast, fungi and other species, e.g. Ure2p-URE3, Sup35p-ERF3-PSI+, Rnq1p-PIN+, New1p-NU+, Prb1p-[vacuolar protease-B], HET-s-[Small-s]. In latter HET-S-[Big-S] allelic variants, different domain patterns comprise accessory R/K-zipper. Sequences and positions suggest that only some EPA are transmissible, confined to species barriers or prone to *"bioaptamer disease"*. CONCLUSIONS: Since not directly retranslatable to heritable genome codes by sequence edition, base modification, redox- and metalloregulation, i.e. code extensions/alterations, entitled *"non-coding"* RNA bioaptamers as products of overwhelming portions [~98%] of transcriptional output apparently represent codes for EPA. RNA-, redox- and metal ion-orchestrated CPLL are suggested reprogramming cell fate in searched concepts [Wickner et al., Genes Develop.18:470-485,2004]: "Proteins as genes and infectious entities".

2849

**Identification and Characterization of a Novel Protein Associated with Early-Onset Dystonia Protein TorsinA**

L. M. Imboden, L. Chin, L. Li; Department of Pharmacology, Emory University School of Medicine, Atlanta, GA

Early-onset generalized dystonia (DYT1) is a movement disorder characterized by involuntary movements and prolonged muscle contraction, resulting in twisting body motions, tremor, and abnormal posture. DYT1 has been linked to two mutations (ΔE302 and ΔF323-Y328) in the AAA+ protein torsinA. To identify additional players in the torsinA pathogenic pathway, we performed yeast two-hybrid screens to search for torsinA-interacting proteins in the brain. Here we report the identification of a novel protein named printor (protein interactor of torsinA). Printor is a 70-kDa protein with no apparent signal sequence or transmembrane domain. Western blot analysis reveals that printor is expressed in brain as well as other tissues, suggesting that printor has a functional role important to many cell types, including neurons. Subcellular fractionation studies indicate that printor exists in both cytosolic and membrane-associated pools. Immunofluorescence confocal microscopic analysis demonstrates that, like torsinA, printor is localized to endoplasmic reticulum and nuclear envelope, but not early endosome, lysosome, or mitochondria. We have confirmed the interaction of printor with torsinA by coimmunoprecipitation and shown that printor colocalizes with torsinA in cells by double immunofluorescence labeling. These results provide compelling evidence that printor and torsinA associate *in vivo* and suggest a potential role for printor in the molecular pathogenesis of DYT1. Supported by NIH Grant NS047575

2850

**Mutations Identified in Myoclonus-Dystonia Patients Alter Epsilon Sarcoglycan Protein Localization**

L. LIU, L. J. Ozelius; Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY

Epsilon-sarcoglycan (SGCE) is an N-glycosylated transmembrane protein expressed ubiqitously. It is functionally related to the other sarcoglycans including alpha-, beta-, delta-, gamma- and zeta- sarcoglycans. Evidences showed a tight interaction of beta-, delta- and epsilon-sarcoglycan in the dystroglycan complex in muscles. Mutations in the SGCE gene cause Myoclonus Dystonia (M-D), a neurologic movement disorder characterized by lightening-like jerks (myoclonus) and sustained twisting and repetitive movements, resulting in abnormal postures (dystonia). The function of SGCE remains unknown. Using site directed mutatgenesis, SGCE mutations found in M-D patients were generated, cloned into a mammalian expression vector and expressed in neuronal like cell lines. Immunohistochemical analysis of wild type and mutant SGCE protein showed significant differences in the localization patterns in the cells with the mutant proteins failing to be transported to the membrane. Protein expression studies suggest that various mutant SGCE proteins interact with wild type protein and partially interfering with targeting of the wild type protein to the membrane. Studies to determine whether the tight interaction of beta-, delta- and epsilon- sarcoglycan might be disrupted due to the mislocalization of mutant SGCE proteins are ongoing. Characterization of mutant SGCE protein will help to better understand the mechanism of myoclonus dystonia and may suggest possible treatment strategies.

2851

**Drug Screening for Potential Therapeutic Development in a *C elegans* Dystonia Model**

A. K. Clark, S. Cao, K. A. Caldwell, G. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL

Dystonia is a human movement disorder that is estimated to affect over 500,000 people in North America and is more prevalent then Huntington's Disease, ALS, and Muscular Dystrophy - combined. The most severe, early-onset form of dystonia is a dominant disorder caused by a single codon deletion in a gene termed *DYT1 (TOR1A)* that encodes a protein called torsinA. A logical route toward therapeutic intervention for dystonia involves the identification of small molecules that have the ability to alter the function of mutant torsinA. We have established the nematode *C. elegans* as a model system by which the activity of torsins can be examined in the context of their putative role as molecular chaperones. Previous work has shown that torsin proteins have the capacity to suppress misfolding of proteins and can protect dopaminergic neurons from toxic insults such a 6-hydroxydopamine treatment in worms. We are conducting a screen for compounds that alter the functional consequences of co-expressing both wildtype and mutant human torsinA in vivo. Here we report use of a set of 240 chemically diverse small molecules, representing off-patent drugs approved for human use, and prescreened for toxicity to *C. elegans*, that we have assayed for reversal of mutant torsin activity in two distinct assays for bioactivity in altering: 1) protein misfolding and 2) dopaminergic neurotoxicity. We have identified 8 drugs that show over 20% increase in blocking protein misfolding and 3 compounds that exhibit a similar enhancement of neuroprotection. 2 of these compounds were overlapping in both assays. These are being examined for torsin-specific effects and will be further investigated as potential lead compounds for chemical modification toward therapeutic development.

2852

**The SPG3A Hereditary Spastic Paraplegia Protein Atlastin-1 is Enriched in Axonal Growth Cones**

P. Zhu,[1] S. J. H. Tao-Cheng,[2] C. L. Smith,[3] J. Stadler,[1] S. M. Yang,[1] C. Blackstone[1]; [1]Cellular Neurology Unit, NIH / NINDS, Bethesda, MD, [2]NINDS Electron Microscopy Facility, NIH / NINDS, Bethesda, MD, [3]NINDS Light Imaging Facility, NIH / NINDS, Bethesda, MD

Mutations in the atlastin-1 gene have been identified in SPG3A, one of the more common forms of autosomal dominant “pure” hereditary spastic paraplegia (HSP), likely resulting in the functional impairment of the atlastin-1 protein. The HSPs are a group of over 25 inherited neurological disorders with the cardinal feature of lower extremity spasticity and weakness resulting from a length-dependent axonopathy of the upper motor neurons. An interesting feature of SPG3A is its very early onset, raising the possibility that developmental abnormalities may be involved in its pathogenesis. We have previously found that atlastin-1 is a large oligomeric GTPase in the dynamin superfamily that is an integral membrane protein localized predominantly to the *cis*-Golgi apparatus in neurons (Zhu et al., J Biol Chem 278:49063-71, 2003), though smaller amounts are also found in the endoplasmic reticulum (ER). Yeast two-hybrid, gel exclusion chromatography, and chemical cross-linking studies have revealed that SPG3A mutations do not affect formation of atlastin-1 oligomers, indicating that these mutant subunits may interfere with the atlastin-1 GTPase complex function in a dominant-negative fashion. Immunoblotting experiments reveal extensive expression of atlastin-1 in the brain and spinal cord in rats, and the level of expression in brain increases from embryonic through adult ages. Unexpectedly, using confocal and electron microscopy we have also found that atlastin-1 is highly enriched in vesicles within axonal varicosities and growth cones in rat cortical neurons in culture, prefiguring a functional role in axonal development. On the other hand, multiple protein markers for the Golgi apparatus and ER are not detected in these compartments. Thus, the "long axonopathy" in SPG3A may result from abnormal ER-Golgi membrane trafficking and/or the

proper development of axons.

2853

**Localization of Spastin and Spartin to Cellular Organelles, and Analysis of their Interaction Proteins**

J. Lu, P. C. Byrne; Pathology, UCD, Dublin, Ireland

Hereditary spastic paraplegia (HSP) describes a diverse group of disorders characterized by length dependent axonal degeneration that causes progressive paralysis affecting the lower limbs. HSP is due to mutations in a number of genes, including SPG4, which encodes spastin and SPG20 which encodes spartin. Both proteins have an MIT (microtubule interacting and trafficking) domain in the amino terminal of the protein and an AAA (ATPases Associated with diverse cellular Activities) domain in the carboxy terminal. Through subcellular localisation studies of fluorescent tagged full-length, NH2-terminal deleted and prematurely truncated spastin and spartin, we identified a novel site of spastin expression -the endoplasmic reticulum, and the localization of spartin in mitochondria. The findings were not reported previously. We have also confirmed that spastin is also expressed in multiple cellular locations including endosomes. Using NH2-terminal deleted and prematurely truncated spastin/spartin constructs we found that sequences at the NH2-terminal region of spastin and C-terminal region of spartin contain the primary determinants for ER and mitochondria localisation respectively. Through acceptor quenching FRET analysis of cultured cells co-expressing EYFP -alpha tubulin and ECFP-spastin, we showed the interaction of tagged spastin and spartin with the alpha-tubulin, indicating the interaction of these proteins with microtubule structures.

2854

**Axons Sever to Survive: Spastin, Atlastin, and Microtubule Severing in Hereditary Spastic Paraplegia**

B. P. Lauring; Pathology, Columbia University, New York, NY

Mutations in the AAA ATPase Spastin (SPG4) cause an autosomal dominant form of hereditary spastic paraplegia (HSP) a retrograde axonopathy primarily characterized pathologically by degeneration of long spinal neurons in the corticospinal tracts and the dorsal columns. Using purified components with real-time videomicroscopic and sedimentation assays we show that recombinant Spastin severs taxol-stabilized microtubules. We find that six mutant forms of Spastin including three disease-associated forms are severely impaired in ATPase and severing activities. Like other AAA ATPases, Spastin oliogomerizes in response to ATP, and some disease-associated mutations abolish assembly whereas others may prevent disassembly. Using a yeast two hybrid screen we identified Atlastin, another HSP gene encoding a golgi-localized integral membrane protein GTPase, as a Spastin binding protein. We show that the two recombinant proteins bind one another in vitro, can be co-immunoprecipitated from cell lysates, and that one protein appears to localize the other in cells. The domain of Spastin binding Atlastin is dispensable for microtubule severing, and recombinant Atlastin does not alter the ATPase activity of Spastin. Instead, Atlastin may recruit Spastin to the golgi apparatus. In cultured hippocampal neurons Spastin expression appears to result in increased dendrite numbers and branching, perhaps indicating a role for microtubule severing in branch formation. We further show that a putative C. elegans orthologue of Spastin indeed severs microtubules and that motor axons terminate prematurely in C. elegans null for Spastin. These studies suggest that defects in microtubule severing are a cause of axonal degeneration in human disease, and that multiple HSP genes may function in a common cell biological pathway important in axonal maintenance.

2855

**Gemin4: A Potential Modifier of Spinal Muscular Atrophy?**

M. P. Walker, K. B. Shpargel, A. G. Matera; Genetics, Case Western Reserve University, Cleveland, OH

Spinal muscular atrophy (SMA) is the leading monogenetic cause of infant morbidity and mortality. Deletion and/or mutations of the survival of motorneurons gene 1 (SMN1) causes SMA. SMN is part of a macromolecular structure known as the SMN complex which is involved in the biogenesis of the essential pre-mRNA splicing factors, the UsnRNPs. The SMN complex is comprised of SMN and seven other core proteins, collectively called Gemins. SMN has been extensively characterized because of it's involvement in SMA. However, detailed functional studies of the other members of the SMN complex are lacking. Our lab is interested in deciphering the role of these Gemin proteins in the complex which in turn may better help us to understand the molecular mechanisms involved in SMA disease. Here we describe the creation and characterization of a mouse model of Gemin4. Gemin4 is a 1058 aa polypeptide that has no known homology to other proteins. Initial studies of Gemin4 revealed that it interacts with the SMN complex via its direct contact with the putative RNA helicase, Gemin3. We have found that disruption of Gemin4 function is lethal. No homozygous mutant neonatal (P1) or mid-gestation (E13.5) animals were recovered, indicating an early embryonic lethal phenotype. We are currently analyzing early-gestation embryos and investigating Gemin4's potential as a modifier of the SMA phenotype. These results indicate that Gemin4 is an essential member of the SMN complex.

2856

**Role of the Survival of Motoneuron (SMN) Protein in Neurite Outgrowth**

J. van Bergeijk,[1] A. F. Bruns,[1] C. Grothe,[1,2] P. Claus[1,2]; [1]Neuroanatomy, OE 4140, Hannover Medical School, Hannover, Germany, [2]Center for Systems Neuroscience (ZSN) Hannover, Hannover, Germany

Spinal muscular atrophy (SMA) is a neurodegenerative disease affecting motoneurons in the spinal cord. SMA is caused by mutations or a deletion of the survival of motoneuron (SMN) gene 1. SMN has a functional role as an assembly protein for small nuclear ribonucleoprotein particles (snRNP) involved in splicing. SMN dissociates from snRNPs in the nucleus and localizes to certain nuclear bodies called nuclear gems. Patients with SMA display significantly less or no nuclear gems. In this study, we performed a functional domain mapping of the SMN protein with respect to differential nuclear localization and effects on neurite outgrowth in PC12 cells. SMN deletions were fused to green fluorescent protein (GFP) and their localization analyzed. A mutant without the C-terminus lost the ability to form nuclear gems and located instead diffusely to the nucleoplasm. In the neurite outgrowth assay, full-length SMN enhanced neurite outgrowth. Interestingly, a mutant displaying the C-terminus only demonstrated the same effect, whereas its ability to assemble snRNPs was impaired. These data argue for a splicing-independent contribution of SMN to neurite growth. We have recently shown a direct interaction between SMN and the neurotrophic fibroblast growth factor - $2^{23}$ (FGF-$2^{23}$). FGF-2 is not only an extracellular signaling molecule, but also a protein found in the nucleus. With regard to function, this interaction suppresses the promoting effect of SMN on neurite outgrowth in PC12 cells. Interestingly, on a molecular level FGF-$2^{23}$ can compete with Gemin2, a

component of the SMN complex in nuclear gems, for binding to SMN. As a functional consequence on a cellular level, the number of nuclear gems is significantly decreased. The results demonstrate the importance of the C-terminus of SMN for regulation of neurite outgrowth and a possible involvement of FGF-2 in this process.

2857

**Visualization of Dimer/Oligomer Formation by Polyglutamine Stretches in Living Cells**

T. Takahashi,[1] S. Kikuchi,[2] M. Nishizawa,[2] O. Onodera[3]; [1]Health Science, Faculty of Medicine, Niigata University, Niigata, Japan, [2]Neurology, Brain Research Institute, Niigata University, Niigata, Japan, [3]Clinical Neuroscience Branch, Brain Research Institute, Niigata University, Niigata, Japan

Polyglutamine diseases are inherited neurodegenerative disease with intranuclear aggregate formation caused by the expansion of a homopolymeric glutamine stretch in various genes. Recent works revealed that the aggregate formation showed protective activity against expanded polyglutamine cytotoxicity. Therefore one of the hypotheses is intermediates, such as dimers or oligomers of expanded polyglutamine stretches might cause cell dysfunction. To clarify this possibility, it is important to visualize the dimer or oligomer formation in living cell. For this purpose, we generated fusion proteins of monomeric-YFP(mYFP) or monomeric-CFP(mCFP) and polyglutamine stretch. We cotransfected COS7 or SH-SY5Y with these in several different combinations. If two fluorescent proteins are close enough to produce fluorescence resonance energy transfer (FRET), we can detect them by fluorescence confocal microscopy. FRET was only observed in combination with mCFP-poly(Q) / mYFP-poly(Q) or poly(Q)-mCFP / poly(Q)-mYFP. The number of the cells which showed FRET increased in a length dependent manner (Q12-9.5%, Q36-24.2%, Q56-27.2%, Q80-36.7%). By construct, in combination with mCFP-poly(Q) / poly(Q)-mYFP or poly(Q)-mCFP / mYFP-poly(Q), we could not detect any FRET. This result indicated that polyglutamine stretch made dimer in a parallel manner, not in an anti-parallel manner in living cell. Furthermore, the intensity of FRET increased in a length dependent manner. It suggested that the stability of dimer formation increased by a length-dependent manner. This new technique to visualize the dimmer/oligomer of expanded polyglutamine stretches in living cell will tell us a new aspect of pathogenesis of polyglutamine diseases.

2858

**Alsin Mediates IGF-1 Receptor Signaling and Cell Survival**

J. D. Topp,[1] S. R. Severson,[1] B. F. Horazdovsky,[1] P. C. Orban,[2] M. R. Hayden,[2] R. S. Devon[2]; [1]Biochemistry & Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, [2]Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada

Amyotrophic lateral sclerosis (ALS) is a heterogeneous group of neurological disorders that result in motor neuron degeneration and usually leads to death. Although ALS is predominantly sporadic, familial ALS accounts for approximately 10% of patients with this illness. Eight independent chromosomal loci have been linked to ALS, and three specific genes have been identified that are mutated in familial forms: *SOD1, SETX* and *ALS2. ALS2* is mutated in a recessive juvenile-onset form of ALS and has also been linked to juvenile primary lateral sclerosis (JPLS) as well as infantile-onset ascending hereditary spastic paraplegia (IAHSP). We have previously shown that the *ALS2* gene product (Alsin) is a Rab5 and Rac1 guanine nucleotide exchange factor (GEF). In an effort to understand the molecular basis of juvenile ALS, we have chosen to examine the functional role of Alsin in neuron maintenance and survival using a cell culture model system. Insulin-like growth factor 1 (IGF-1) signaling was significantly impaired in cells expressing mutant Alsin lacking an intact Rab5 GEF domain. Though IGF-1 receptor activation and internalization were unaffected, trafficking of activated receptors to early endosomes was severely compromised. Since IGF-1 plays an important role in mediating cell survival, we examined IGF-1 anti-apoptotic activity in cells expressing the mutant form of Alsin. The IGF-1 signaling defect observed in Alsin defective cells also resulted in reduced IGF-1 protection from serum withdrawal-induced apoptosis. We conclude that mutant Alsin proteins compromise IGF-1 receptor trafficking, IGF-1 signaling, and IGF-1 mediated cell survival, identifying a potential mechanism for motor neuron degeneration observed in juvenile ALS.

2859

**Identification of Chemical Compounds that Rescue the BDNF Vesicular Transport Defects in Huntington's Disease**

J. P. Dompierre, J. Godin, B. Charrin, S. Humbert, F. Saudou; Cnrs umr 146, Institut Curie, Orsay, France

Huntington's disease (HD) is a fatal neurodegenerative disorder that is characterized by involuntary movements, personality changes and dementia. The neuropathology of HD involves neuronal dysfunction and the selective death of striatal neurons in the brain. The mutation that causes disease is an abnormal expansion of a polyglutamine (polyQ) stretch in the N-terminus of the 350 kD protein huntingtin. The mechanisms by which huntingtin induces dysfunction and death of neurons in the brain are not clearly understood. These could involve the gain of a new toxic function as well as the loss of the beneficial activities intrinsic to wild type huntingtin. Indeed, huntingtin possesses anti-apoptotic properties as observed in cell culture and animal models. We have used 3D fast video microscopy techniques to study the intracellular dynamics in normal and pathological situations. Using this approach we have unravelled a function of huntingtin in the microtubule-based transport of neurotrophic factors such as BDNF. In the pathological situation, huntingtin-stimulated BDNF transport is altered. We demonstrated that polyQ-huntingtin *via* the huntingtin-associated protein-1, HAP1 disrupts the association of key components of the motor machinery to microtubules. Reduced BDNF transport leads to a decrease in neurotrophic support and to neurotoxicity that are both rescued by wild-type huntingtin. Our results demonstrate that the anti-apoptotic properties of huntingtin are linked to the ability of huntingtin to promote transport of BDNF in the brain. We will report recent findings that identify compounds that rescue huntingtin's dysfunction in intracellular dynamics and how they act to inhibit the toxicity of mutant polyQ-huntingtin in disease.

2860

**Glucocorticoid Receptor Antagonists Are Effective in Eliminating Allodynia and Hyperalgesia in Mouse Model of Neuropathic Pain**

T. Tanabe, I. Takasaki, T. Kurihara, H. Saegusa, S. Zong; Pharmacology & Neurobiology, Tokyo Medical & Dental University Graduate School of Medicine, Tokyo, Japan

We have previously demonstrated that mice lacking N-type voltage-dependent Ca channels (VDCCs) show markedly reduced symptoms of

neuropathic pain-related behavior induced by spinal nerve injury, suggesting the critical role of N-type VDCCs in the development of neuropathic pain. Although several lines of studies demonstrated that blockade of this channel is effective on neuropathic pain, it has been reported that clinical application of an N-type VDCC blocker has a limitation due to its serious side effects. Our results of cDNA microarray analysis suggest that glucocorticoid receptor (GR) mRNA is up-regulated in the spinal cord of mice with spinal nerve injury as compared with sham operated mice, whereas GR mRNA expression is decreased in N-type VDCC deficient mice by nerve injury. These results suggest that GR in the spinal cord contributes to the development and/or maintenance of neuropathic pain induced by nerve injury. GR is widely expressed in the central nervous system including the regions involved in the control of pain transmission. However, the contribution of GR to neuropathic pain remains unknown. Thus we examined the effects of GR antagonists on neuropathic pain-like responses in mice. Injury to spinal nerves of mice induced mechanical allodynia and thermal hyperalgesia, and these responses were ameliorated by intrathecal injection of the GR antagonists. Intraperitoneal injection of GR antagonists also produced antinociceptive effects, whereas intracerebroventricular injection was without effects. These results suggest that spinal GR plays important roles in neuropathic pain, and controlling the activity of GR can be of great importance for the treatment of neuropathic pain.

2861

### Intracellular Calcium-activated Protein Kinase $C_{\beta II}$ and Mitochondrial $Ca^{2+}$-mediated Oxidative Stress Are Essential for the Geldanamycin-Induced Synthesis of GRP78

F. Sun, H. Shyn, Y. Lai; National Tsing Hua University, Hsinchu, Taiwan Republic of China

The molecular mechanism whereby antitumor drug geldanamycin (GA) impacts ER stress pathway is partially known to be involved with the activation of calcium, oxidative stress and protein kinase C. Here, we investigated the roles of calcium, oxidative stress and their causal relationship in GA-induced expression of ER chaperone GRP78 in 9L rat brain tumor cells. GA treatment leads to an immediate suppression of general protein synthesis followed by an enhanced synthesis of the GRP78. GA-exerted calcium signaling was completely blocked by altering mobilization of calcium in organelles with intracellular or extracellular calcium chelator, BAPTA/AM and EGTA. GA-induced increase of $[Ca^{2+}]_i$ is also 50% prevented in the presence of thapsigargin (TG) and ruthenium red (RuR). These data lead us conclude that extracellular $Ca^{2+}$ are the major sources of GA-evoked calcium signaling, and the calcium store in ER and mitochondria are also involved in the process. Furthermore, U73122 as a phospholipase C inhibitor decreased GA-induced GRP78 expression, suggesting the dependence of phospholipase C in the process. In specific kinase inhibitors screening assay, we identify $PKC_{\beta II}$ as a possible PKC subtype specifically involved in GA-induced synthesis of GRP78. GA-induced ROS formation was shown to be dependent on the presence of extracellular $Ca^{2+}$ and was further reduced when cytosolic $Ca^{2+}$ was chelated by BAPTA/AM and EGTA. To delineate the causal effects, we showed that the increase of intracellular calcium activating $PKC_{\beta II}$ preceds the generation of ROS. We further concluded that inductive GRP78 expression by GA requires consecutive mediators of calcium, $PKC_{\beta II}$ and ROS to concert this induction.

2862

### Crosstalk Between Huntingtin N-terminal Fragments and Syntaxin 1A Regulates N-type Calcium Channels

L. Swayne, L. Chen, G. W. Zamponi, J. E. Braun; Physiology and Biophysics, University of Calgary, Calgary, AB, Canada

We have identified a novel interaction between N-terminal huntingtin fragments ($huntingtin^{exon1}$) and the synaptic protein interaction (synprint) region of the $Ca_v2.2$ subunit of the N-type calcium channel, which is a voltage-gated calcium channel important for neurotransmission. The synprint region associates with $huntingtin^{exon1}$ containing both wildtype and mutant numbers of glutamine repeats and the native proteins interact in mouse and brain homogenates. The interaction of $huntingtin^{exon1}$ with N-type calcium channels results in the disruption of syntaxin 1A-mediated effects on both channel inactivation and regulation by G proteins. To begin to address the mechanism through which htt disrupts syntaxin 1A effects on the N-type channel, we examined the $huntingtin^{exon1}$- and syntaxin 1A-binding properties of $Ca_v2.2$ synprint, and demonstrate that these proteins bind to overlapping sites of $Ca_v2.2$ synprint. We further show that $huntingtin^{exon1}$ and syntaxin 1A compete for synprint binding *in vitro*, suggesting that huntingtin exerts its effects through competition with syntaxin 1A for synprint binding. These results identify the regulation of N-type calcium fluxes as a novel role of huntingtin.

2863

### Signaling Pathway of Ginsenoside-Rg1 Leading to Nitric Oxide Production in Endothelial Cells

K. Leung,[1] N. Mak,[1] T. Fan,[2] R. Wong[1]; [1]Department of Biology, Hong Kong Baptist University, Hong Kong, China, [2]Department of Pharmacology, University of Cambridge, Cambridge, United Kingdom

The pharmacological activity of *Panax ginseng* has been ascribed to a group of steroidal saponins known as ginsenosides, of which more than 30 members have been isolated and characterized from the *Panax* species. We here provide evidence that Rg1, one of the major ginsenosides of ginseng, is a functional ligand of the glucocorticoid receptor as determined by fluorescence polarization assay ($IC_{50}$ = 140 nM; $K_d$ = 43 nM). Rg1 increased the phosphorylation of the glucocorticoid receptor, phosphatidylinositol-3 kinase, Akt/protein kinase B, endothelial nitric oxide synthase (eNOS) and NO output in human umbilical vein endothelial cells. Phosphorylation of eNOS and the production of nitric oxide were significantly reduced either by RU486 (glucocorticoid receptor antagonist), LY294002 (phosphatidylinositol-3 kinase inhibitor), SH-6 (Akt/protein kinase B inhibitor), or L-NMMA (nitric oxide synthase inhibitor). The Rg1-induced phosphorylation and the subsequent translocation of the glucocorticoid receptor to the nucleus were also abolished by RU486. This study revealed that Rg1 can indeed serve as an agonist ligand for the glucocorticoid receptor and the activated glucocorticoid receptor can induce rapid nitric oxide production from endothelial nitric oxide synthase via the non-transcriptional phosphatidylinositol-3 kinase /Akt pathway.

2864

### The Inhibition of Glucose Transporter-4 (Glut4) by Down Syndrome Critical Region-1 (DSCR1)

S. Lee, H. Youn; Biochemistry and Molecular Biology, Seoul National University College of Medicine, Seoul, Republic of Korea

Down syndrome (DS) has been caused by trisomy of human chromosome 21. The study of patients with partial trisomy 21 has defined an area of approximately 3 Mb at chromosomal region 21q22 as Down syndrome candidate region. In particular, since the gene of Down syndrome critical

region-1 (DSCR1) is located in human chromosome 21q22.1-q22.2 and encodes a 197aa-long protein, DSCR1 has been suggested as one of candidate genes involved in DS. It was initially proposed that DSCR1 negatively regulated the calcineurin activity because DSCR1 was found as a calcineurin inhibitory protein. On the other hand, an increasing number of degenerative diseases, including DS, are known to be associated with increased insulin resistance. Thus we investigated whether DSCR1 influences glucose uptake upon insulin stimulation. Ectopically-expressing DSCR1 markedly inhibited the translocation of Glut4 into plasma membrane, and also inhibited [C]$^{14}$-glucose uptake upon insulin stimulation. When the effective domain of DSCR1 was narrowed down, N-terminal domain of DSCR1 (1-90 amino acid) inhibited the translocation of Glut4. Taken together, we suggest that the intrinsic function of DSCR1 may negatively regulate Glut4 translocation to block glucose uptake in neuronal cell.

2865

**Myelin Protein Zero Trafficking and Quality Control in Charcot-Marie-Tooth Neuropathies**

M. D'Antonio,[1] M. Pennuto,[1] E. Tinelli,[1] A. Quattrini,[2] M. L. Feltri,[1] L. Wrabetz[1]; [1]San Raffaele Scientific Institute, DIBIT, Milano, Italy, [2]Department of Neurology, Ospedale San Raffaele, Milano, Italy

Myelin is a multilamellar membrane that surrounds and supports axons, and facilitates rapid saltatory conduction of impulses. Myelin Protein Zero (MPZ, P0) is the most abundant glycoprotein of peripheral myelin. Several human inherited neuropathies with defects in peripheral nerve myelin are caused by mutations in the MPZ gene. Diverse mutations produce different phenotypes, ranging from mild Charcot-Marie-Tooth (CMT) disease type 1B to the more severe forms Dejerine-Sottas syndrome (DSS) and Congenital Hypomyelination or even axonal neuropathy, suggesting gain of function mechanisms. Deletion (S63del) or conversion of serine 63 to cysteine (S63C) results in CMT1B or DSS, respectively. We show that when expressed in mouse together with wild type P0, either mutant P0 produces a demyelinating neuropathy that mimics the corresponding human disease. In parallel with diverse nerve phenotypes, S63C is mostly trafficked to myelin where it creates a packing defect, while S63del is retained in the endoplasmic reticulum (ER)-Golgi complex. Protein quality control in the ER ensures that only correctly synthesized proteins are delivered to the final destination. Indeed, accumulation of P0S63del in the ER triggers an unfolded protein response (UPR) in a dose-dependent fashion, indicating a toxic gain of function. Preliminary data suggest that the UPR mediator CHOP transcription factor modulates neuropathy in Ser63del mice. Systematic analysis of gene expression is underway in these mice. These data suggest that different forms of protein quality control contribute to diverse demyelinating phenotypes in CMT neuropathies.

2866

**DISC1 Regulates the Localization of Grb2 into the Axon via Kinesin-1**

T. Shinoda, S. Taya, K. Kaibuchi; Department of Cell Pharmacology, Nagoya University, Nagoya, Japan

*Disrupted-In-Schizophrenia-1* (*DISC1*) is identified at the breakpoint of a chromosomal translocation associated with schizophrenia in a large Scottish family. The physiological functions of DISC1, however, have been not well understood. Here, we identified Grb2 as a novel DISC1-interacting molecule by using affinity column chromatography. Grb2 is crucial for the activation of various intracellular signaling pathways. Grb2 is composed of one Src-Homology-2 (SH2) domain and two Src-homology-3 (SH3) domains. The N-terminal SH3 domain of Grb2 directly recognized DISC1. In cultured hippocampal neuron, both DISC1 and Grb2 accumulated in the distal part of axon. The knockdown of DISC1 by RNA-interference resulted in the delocalization Grb2 from axonal tips. As we previously identified Kinesin-1 as a DISC1-interacting molecule, we investigated the possibility that DISC1 is involved in the Grb2 transport or localization via Kinesin-1. Kinesin-1 is a molecular motor on microtubules, composed of two kinesin heavy chains (KIF5A, 5B or 5C) and two kinesin light chains (KLC1 or KLC2). Grb2, DISC1 and KIF5A formed a trimolecular complex both in vivo and in vitro. The functional blocking of Kinesin-1, by using a dominant-negative form of KIF5A or by the knockdown of kinesin light chains, resulted in the misloalization of Grb2 as well as DISC1 from the distal part of axon. These results suggest that the localization of Grb2 is regulated by DISC1 and Kinesin-1.

2867

**Mimicking in-vivo Conditions at the Rim of an Ischemic Infarct Core**

H. Yao,[1] Y. Shu,[2] G. G. Haddad[1]; [1]Department of Pediatrics, Albert Einstein College of Medicine, Bronx, NY, [2]Department of Neurobiology, Yale University School of Medicine, New Haven, CT

Acute reduction of blood supply to an area in the brain can create immediately an ischemic infarct, a core that harbors dead cells. Due to processes of release or secretion from dead or dying cells, the biochemical and ionic alterations within the core are dramatic. Hence, adjacent cells are generally exposed to these changes and may be affected deleteriously, enlarging the core into the penumbra. To develop an in-vitro model that mimics some of these conditions, we have developed an ischemic solution (IS) that resembles the extracellular fluid in the infarct core “territory”. Compared to normal solutions (ACSF), IS is characterized by a) low $O_2$ (1.5%); b) low glucose (3 mM); c) low pH (6.4); d) excitotoxic (100 µM glutamate); and e) ionic alterations (64 mM $K^+$, 51 mM $Na^+$, 0.13 mM $Ca^{2+}$, 81.5 mM $Cl^-$ ). We have examined cell injury/death using propidium iodide (PI) uptake in organotypic hippocampal slice cultures after exposure to IS. Our preliminary data have shown a significant PI uptake (mean fluorescent intensity: MFI) starting 4 hours after IS treatment in CA1, CA3 and DG regions in hippocampal slices (for example, MFI in CA1: 67±5, n=36 in IS group vs 38±2, n=19 in ACSF group, $p < 0.001$). PI uptake continued to increase with continued exposure (at 24 hour for example, MFI in CA1: 596±34, n=36 in IS group vs 192±23, n=20 in ACSF group, $p < 0.001$). These data suggest that a direct exposure to IS can cause neuronal degeneration and the underlying mechanisms of cell death may resemble those in the territory between the infarcted core and the penumbral area following focal ischemia.

2868

**Inactivation of Calcineurin by Redox-mediated Proteolysis**

J. Lee, H. Kim, H. Youn; Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, Seoul, Republic of Korea

An increase in reactive oxygen species (ROS) is correlated with the loss of calcineurin activity under oxidative or neuropathologic conditions. However, the detailed molecular mechanism of ROS-mediated calcineurin inactivation is still obscure. Here we describe a novel mechanism by

which hydrogen peroxide inactivates calcineurin by cleaving the specific sequence within the catalytic domain. The cleavage form of calcineurin completely lost its enzymatic activity. The post-treatment of antioxidant *in vivo* did not recover the calcineurin activity damaged by hydrogen peroxide, suggesting that hydrogen peroxide irreversibly inactivates calcineurin *in vivo* by proteolytic cleavage rather than by the alteration of redox state of metal center. Furthermore, calcineurin was markedly cleaved in brain tissues of Alzheimer disease patients. Thus, our findings provide a new insight into how redox insult inactivates calcineurin and accumulates hyperphosphorylated tau in Alzheimer disease.

2869

**RING Finger Protein 11 Localizes to Mitochondria and Interacts with Mitochondrial Proteins**

B. R. Whatley, L. Chin, L. Li; Pharmacology, Emory University, Atlanta, GA

Dysregulated ubiquitin/proteasome-mediated protein degradation and impairment of mitochondrial function have both been implicated in the pathogenesis of several neurodegenerative diseases, including Parkinson's and Alzheimer's disease. However, the interplay between these two processes is not understood. Among the molecular components of the ubiquitin-proteasome pathway, the E3 ubiquitin-protein ligase is perhaps the most important player because it recruits the substrates for ubiquitination and determines the specificity of protein degradation. RING finger protein 11 (RNF11) is a ubiquitously expressed protein originally identified in a search for novel proteins containing the RING finger, a motif that has been implicated as a key determinant of the E3 ligase activity. The biochemical function and cellular role of RNF11 remain poorly characterized. To identify protein interactors of RNF11, we performed yeast two-hybrid screens of a rat brain cDNA library using full-length and C-terminally truncated (deletion of the RING finger motif) forms of RNF11 as bait. We isolated two different clones that encode mitochondrial proteins. Immunofluorescence confocal microscopic analysis reveals that, in transfected HeLa cells, RNF11 exhibits significant colocalization with mitochondrial markers TIM23 and Mitotracker. To confirm the mitochondrial localization of RNF11, crude mitochondrial fractions were prepared from lysates of transfected HeLa cells and further fractionated on a 10-30% OptiPrep gradient. Immunoblot analysis of the obtained fractions shows clear co-fractionation between RNF11 and mitochondrial markers. These results suggest that RNF11, a putative E3 ubiquitin-protein ligase in brain, may play a role in the regulation of mitochondrial function.

2870

**Manipulating the Intracellular Fate of a Protein: Making an Aggregate-Prone Mutant Rhodopsin to Fold**

S. Kaushal, B. Noorwez, S. Noorwez; Ophthalmology, University of Florida, Gainesville, FL

Like many other acquired and genetic protein conformational diseases (PCDs), P23H opsin associated with Autosomal Dominant Retinitis Pigmentosa (ADRP) is a misfolded protein retained within the cell and forms inclusion bodies. We had previously described a set of pharmacological chaperones, including the native chromophore 11-*cis* retinal, that promoted the *in vivo* folding and stabilization of P23H opsin. Like the wild-type protein, the rescued mutant formed pigment and was transported to the cell surface. In this study, we investigate the effect of inhibitors of various cellular enzymes and pathways that may participate in the intracellular fate of P23H opsin. Mutant and WT and P23H opsins were expressed separately in HEK293 stable cell lines in the presence or absence of 11-*cis* retinal and various inhibitors. At 48 hrs, the amounts of folded protein were selectively immunoaffinity purified and quantitated by UV-visible spectroscopy. Strikingly, we demonstrate that inhibitors of the proteasome or autophagy led to significant increases (2.5-4.0 X) in the amount of folded P23H rhodopsin while their effect on the WT protein was minimal. We also demonstrate that by inhibiting the endoplasmic reticulum (ER) glucosidases I and II with castanospermine or inhibiting ER to Golgi transport by Brefeldin A increased the yields of the folded mutant protein by 1.2-2X. This is the first demonstration that by titrating the inhibition of various cellular processes, including the major quality control systems of the cells, the amount of a mutant folded protein can be increased to wild-type levels. Further, we clearly establish the importance of both major degradative pathways in the steady-state levels of a mutant protein. In conjunction with chemical and pharmacological chaperones, this approach may be useful as an adjuvant therapy for PCDs like ADRP.

2871

**CLN3 and CLN6 Mutations Lead to Trafficking Defects in Lysosomal-targeted Pathways**

Y. Cao, J. A. Espinola, M. E. MacDonald, S. L. Cotman; Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA

The *CLN3* and *CLN6* genes encode novel membrane proteins disrupted in two forms of neuronal ceroid lipofuscinosis (NCL), juvenile and variant late-infantile NCL, respectively. The *CLN3*-encoded protein, CLN3 or battenin, localizes to endosomal/lysosomal membranes, and the *CLN6*-encoded protein, CLN6 or linclin, localizes to the endoplasmic reticulum membrane. Despite these differing subcellular localizations, both forms of NCL exhibit autolysosomal accumulation of subunit c of the mitochondrial ATPase $F_0$ complex, suggesting battenin and linclin may function in the autophagic turnover of mitochondria. To further study the effects of the *CLN3* and *CLN6* NCL mutations on the autophagic pathway and other membrane trafficking pathways, we have established genetically accurate cerebellar neuronal precursor cell lines from *Cln3*$^{\Delta ex7/8}$ knock-in mice and *Cln6*$^{nclf}$ spontaneous mutant mice. We have previously shown that homozygous Cb*Cln3*$^{\Delta ex7/8}$ cerebellar cells exhibit defects in endocytic membrane trafficking, and more recent studies indicate an upregulation of autophagy. Moreover, changes are observed in two enzymes whose deficiencies also lead to autolysosomal subunit c accumulation, specifically the cathepsin D and tripeptidyl peptidase-I (TPP-I) enzymes. In preliminary studies of homozygous Cb*Cln6*$^{nclf}$ cerebellar precursor cells, we likewise have observed changes in cathepsin D and TPP-I enzymes. In the case of cathepsin D, both battenin and linclin deficiency lead to altered processing of the enzyme. However, TPP-I enzyme is altered differently in each case, with battenin and linclin deficiency leading to increased or decreased levels, respectively. These data support the hypothesis that the *Cln3* and *Cln6*-encoded proteins act at different levels in the same pathway, a notion consistent with their different subcellular localizations and the variable but related NCLs to which they are linked. Future studies will provide further comparative analyses and will further explore the specific membrane trafficking pathways that require battenin and linclin function.

2872

**Glutamate-induced Oxidative Stress Activates a Novel Cell Death Signaling Pathway Where Extracellular Signal Regulated Kinases-1/2 (ERK1/2) Activation Is Downstream of a Caspase-1-like Protease and Upstream of Calpain Activation**

R. Stetler, D. B. Defranco; Neuroscience and Pharmacology, University of Pittsburgh, Pittsburgh, PA

Glutamate-induced oxidative stress in immature neurons and hippocampal-derived HT22 cells occurs independently of excitotoxicity, as these cells

lack ionotropic glutamate receptors. Previous studies have found that inhibition of the mitogen-activated protein kinase (MAPK) pathway, calcium influx, and caspase-1-like protease(s) are protective against cell death caused by glutamate-induced oxidative stress, and the persistent activation of ERK1/2 is necessary for glutamate-induced oxidative toxicity in HT22 cells. However, the interactions between the cell death machinery responsible for glutamate-induced oxidative toxicity and ERK1/2 activation are as yet unknown. We sought to further characterize proteases activated following glutamate-induced oxidative stress in HT22 cells, and determine their interactions with the MAPK pathway. Inhibition of caspase-1- or -2-like proteases by YVAD.fmk or VDVAD.fmk blocked glutamate-induced toxicity and ERK1/2 activation, suggesting that ERK1/2 activation is downstream of a caspase-1- or -2- like protease. We have also found that calpain activity was significantly increased in HT22 cell lysates at 11h following 5mM glutamate treatment. Furthermore, overexpression of the endogenous calpain inhibitor, calpastatin, in transiently transfected HT22 cells delayed glutamate-induced cell death. However, unlike caspase-1- or -2- like inhibitors, calpain inhibition had no significant effect on phospho-ERK1/2 activation. Inhibition of ERK1/2 activation with U0126 blocked glutamate-induced calpain activation, further corroborating the ERK1/2 requirement for activation of calpain. Taken together, these results suggest that two distinct proteases are involved in ERK1/2-dependent oxidative toxicity, where a caspase-1- or -2-like protease appears to lie upstream of ERK1/2 activation, while calpain activation is triggered by chronically active ERK1/2.

2873

### Receptor Usage of Herpes Simplex Virus Type 1 (HSV-1) for Entry of Neurons

M. Yoon, P. G. Spear; Microbiology-Immunology, Northwestern University Medical School, Chicago, IL

Herpes simplex virus type 1 (HSV-1) can enter cells expressing one of multiple entry receptors, including herpesvirus entry mediator (HVEM), nectin-1, nectin-2 and sites in heparan sulfate generated by specific 3-O-sulfotransferases (3-O-S-HS). The viral ligand for these receptors is glycoprotein D (gD). Given that multiple cell surface receptors can be used by HSV-1 to enter cells, which ones are actually used to infect epithelial cells at the portal of entry and to spread to neurons? Usage of alternative receptors can enable a virus to infect multiple cell types that differ in receptor expression. In this study, we have generated a panel of recombinant viruses with altered receptor usage: viral mutants which cannot use HVEM and 3-O-S-HS as entry receptors, while preserving ability to use nectin-1;viral mutants which cannot use HVEM and 3-O-S-HS, while preserving ability to use nectin-1 and conferring its ability to use nectin-2; viral mutants which can not use nectin-1 and 3-O-S-HS as entry receptors, while preserving ability to use HVEM; viral mutants which can not use nectin-1 as entry receptors, while preserving ability to use HVEM and 3-O-S-HS. Viral mutants that can only use HVEM as an entry receptor were unable to infect human neuronal cell lines, including IMR5 and SH-SY5Y, whereas other viral mutants that can use nectin-1 as an entry receptor can infect these cells, suggesting that nectin-1 is the principal receptors for HSV-1 entry. Recombinant viruses use receptors other than nectin-1 may establish transient infections in neurons, but perhaps not latent infections, and are therefore candidates for development of safe live virus vaccines.

## Other Diseases III (2874-2897)

2874

### Immunization with Pemphigus Anti-idiotype F(ab)'$_2$ Fragments Elicit Anti-epithelial Antibodies in Balb/c Mice

E. Avalos-Díaz,[1,2] G. Pacheco-Tovar,[1] J. Bollain-y-Goytia,[1] E. López-Robles,[1] E. Alvarado-Flores,[2] R. Herrera-Esparza[1]; [1]Immunology, Universidad Autónoma de Zacatecas, Zacatecas, Mexico, [2]Servicios de Salud de Zacatecas, Zacatecas, Mexico

Theoretically the immunization with anti-idiotype antibodies would elicit antibodies capable to recognize epitopes like the original idiotype. In present studies adult Balb/c mice were used to produce anti-epithelial antibodies based in internal images of pemphigus anti idiotype antibodies, which were previously produced in rabbits (Cre anti-idiotype), IgG was digested with pepsin to obtain Cre anti-idiotype F(ab)'$_2$ fragments, which were purified by gel filtration chromatography. Control Balb/c mice were weekly immunized with normal F(ab)'$_2$ fragments; another group was immunized with anti-idiotype F(ab)'$_2$ fragments. Animals immunized with anti-idiotype F(ab)'$_2$ fragments develop anti-epithelial antibodies within two months; they had anti-desmoglein 1 specificity demonstrated by ELISA. These animals had epidermal deposition of IgG along inter-cellular spaces. Control Balb/c mice group was negative. Main observation of this work is that internal images of pemphigus anti-idiotype antibody would elicit anti-epithelial antibodies; this immunological approach can be used to understand pathogenic mechanisms in pemphigus.

2875

### Neutrophil Function Is Not Compromised in Mice with Deficiency of the Shwachman-Diamond Syndrome Gene in Myeloid Lineages

S. Zhang,[1,2] J. Zhong,[2] M. Glogauer,[3] J. Rommens[1,2]; [1]Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON, Canada, [2]Genetics & Genomic Biology, The Hospital for Sick Children, Toronto, ON, Canada, [3]CIHR Group in Matrix Dynamics, University of Toronto, Toronto, ON, Canada

The reported neutrophil deficiencies in Shwachman-Diamond syndrome include problems with cell numbers as well as migration responses to chemotactic agents. A novel and highly conserved gene with poorly characterized function, called SBDS, has been identified as being responsible for this genetic disorder with loss of function mutations. Complete loss of Sbds leads to embryonic lethality, so a gene targeting methodology with a Cre-Lox strategy was used to generate Sbds alleles that could be ablated in selected tissues. To characterize the role of Sbds in neutrophil function, breeding with a transgenic mouse with Cre under control of the Lysozyme M promoter, produced mice deficient in Sbds in their monocyte and myeloid lineages. Sbds was confirmed to be markedly reduced in mature neutrophils isolated from mutant mice by Percoll gradient centrifugation using Western blotting. However, circulating cell counts of blood lineages, including neutrophils, were normal in adult mice. In an in vitro chemotaxis assay, mutant neutrophils also showed a normal response to fMLP. Upon in vivo induction of inflammation, the increase of peripheral neutrophils of mutant mice were found to be comparable to wild type mice. To test migration, penetration and adhesion, a peritoneal inflammation model was obtained by injection with sodium periodate. We observed similar neutrophil accumulation between the mutant and wild type mice. Our results indicate that Sbds is dispensable for mature neutrophil and chemotaxis function, and that deficiency in patients is likely due to early hematopoietic abnormalities.

2876

**Mutant Alpha-1 Antitrypsin Induces Expression of Unfolded Protein Response, Apoptosis and Pro-Inflammatory Genes**

V. S. Mishra,[1] C. Lui,[2] J. Stocks,[1] M. Brantly[1]; [1]Medicine, University of Florida, Gainesville, FL, [2]Pathology, University of Florida, Gainesville, FL

Alpha-1 antitrypsin (AAT) is a 52 KD bean-shaped glycoprotein that is synthesized by the liver and secreted in abundance into the circulation. AAT deficiency is one of the most common inherited risk factors for liver disease in adults and children. The most common mutation in the AAT gene associated with AAT deficiency and liver disease is the Z mutation. The Z mutation is a single base pair substitution of A to G that causes a single amino acid (Glu342Lys) change at the base of the reactive site loop. The mutant Z AAT mis-folds and accumulates in the rough ER of the cell as both polymer and monomer. In this context Z AAT is a classic conformational disease. Mis-folded proteins may induced an unfolded protein response (UPR). We hypothesize that Z AAT also induces a UPR that in turn activates down stream genes associate with inflammation and cell death. To evaluate this hypothesis, we used a cell line derived from the liver of PI*Z individual and AAT-specific siRNA to evaluate UPR & AAT gene expression by quantitative PCR. Positive and negative controls demonstrated the there were not off target effects of AAT siRNA transfection. siRNA knock down of AAT gene expression caused significant reductions in the expression of UPR related genes including ATF6 ($p=0.0001$) and Perk ($p=0.002$) as well as, down stream genes involved in protein folding such as BiP ($p=0.008$) & GP96 ($p=0.02$), innate immunity such as TLR4 ($p=0.03$), NFkB ($p=0.02$) and IRAK $p=0.01$) and apoptosis, CHOP ($p=0.01$). These results establish an important relationship between mis-folding of Z AAT, the UPR, pro-inflammatory responses and cell death.

2877

**Effects of BLX-1060, an Orally Active, Amino-Acid Based Small Molecule, in Rodent Models of Inflammatory Bowel Disease**

A. P. Sen, M. Anumula, H. Vargas, A. Nag, D. Dey, P. Nag, B. Nag; Bexel Pharmaceuticals, Union City, CA

BLX-1060 is a water-soluble, amino-acid conjugated small molecule with anti-inflammatory properties. It does not show any cytotoxicity in different cell types upto 100 uM concentrations. Here we report the effects of BLX-1060 on rodent models of inflammatory bowel disease (IBD) and IL-12, a biomarker for IBD. At 3-100 uM concentrations, BLX-1060 in a dose-dependent manner inhibited LPS-induced IL-12 increase in human peripheral blood mononuclear (hPBMC) cells and in mouse peritoneal macrophages. In an *in vivo* efficacy study mice were fed with dextran sodium sulfate (4% w/v) in drinking water to induce IBD. Disease activity index (DAI), a measure for the severity of IBD, was measured. DAI consists of body weight changes, stool consistency and occult blood. BLX-1060 was administered at 1, 10 and 100 mg/kg/day, PO, for 21 days. BLX-1060 dose-dependently decreased the DAI. Histological evaluation of the colons showed a decrease in the severity of colitis by BLX-1060 compared with the vehicle-treated group. In another *in vivo* efficacy study rats were treated with 30 mg of trinitrobenzene sulfonic acid (TNBS) rectally to induce colitis. BLX-1060 was administered at 100 mg/kg/day, PO for 15 days. BLX-1060 prevented TNBS-induced decrease in body weights. Upon termination the colons were subjected to histopathological evaluation. BLX-1060 was found to decrease the severity of inflammation in the colons. Taken together, these studies conclude that BLX-1060 is a potential new drug for the treatment of inflammatory bowel disease.

2878

***hprt* Locus Permissivity for *in vivo* Inducible Over- and Down-expression through Targeted Transgenesis**

F. Jaisser,[1] C. Fages,[2] R. Willmotte,[2] G. Palais,[1] K. Grisoni,[2] A. Peterson[3]; [1]INSERM, Paris, France, [2]Nucleis, Lyon, France, [3]McGill University, Montreal, PQ, Canada

Random transgenesis has many limitations like positional effects (ectopic expression and variability of transgene expression level). One alternative approach is targeted transgenesis at the permissive *hprt* locus. We are using a powerful technology (Speedy Mouse® Technology) for generating "high throughput" *in vivo* models. This strategy is based on ES cells called BPES cells with high germline transmission potency and selectable targeted transgenesis. First, we have optimized different strategies for conditional transgene expression, *ex vivo* and *in vivo* in ES-derived transgenic models. Conditional and inducible gene expression is achieved with the tetracycline system. Recombined BPES with a bidirectional GR-tetO-LacZ construct (allowing tet dependent expression of the glucocorticoid receptor (GR) and LacZ) have been obtained and stably transfected with a CMV-rTAS2-M2 construct. This led to high, inducible LacZ expression, only in the presence of the Doxycycline, indicating the absence of leakness and efficiency of inducibility. F1 mice were obtained by breeding the MHCtetOFF mouse strain and the mouse chimeras derived from the GR-tetO-LacZ BPES cells. Cardiac LacZ expression is currently compared to those obtained after random integration of the same construct. Second, we developed strategies for conditional and inducible *in vivo* RNAi expression after shRNA targeted transgenesis.. In vivo expression of a CD8α shRNA construct targeted into the *hprt* locus of BPES cells led to a specific 82 % inhibition of CD8α expression in both thymus and spleen in F0 and F1 generations. This is similar to what previously obtained by classical random transgenesis with CD8α shRNA lentivirus. In conclusion, single copy integration of either tet inducible or shRNA constructs through targeted transgenesis in the BPES cells appears to be as efficient as random transgenesis but more poweful in term of time and predictability.

2879

**Steady Shear Flow Suppresses Cytokines-Induced Responses in Endothelial Cells**

L. D. Wang; Institute of Biomed. Sciences, Academia Sinica, Taipei, Taiwan Republic of China

Endothelial cells (ECs) are constantly exposed to blood flow-induced shear stress (SS). Laminar shear stress protects ECs from atherogenesis, whereas disturbed flow occurred near bifurcation predisposes ECs toward inflammation. We have shown that SS suppresses serum- and IL-6-induced Stat3 activity. IFNγ-induced chemokines'expression for T cells attachment was shown to be associated with atherogenesis. In this study, the effect of shear stress (SS) on IFNγ-induced responses in ECs was examined by utilizing a well-defined flow chamber system. ECs exposed to IFNγ triggered JAK/STAT1 activation with the phosphorylation of Tyr701 and Ser727 in STAT1. SS alone had no effect on STAT1 activation in ECs at basal condition. When SS was applied to IFNγ-treated ECs, it significantly attenuated IFNγ-induced Tyr701 phosphorylation with a shear force- and time- dependent manner whiles Ser727 phosphorylation was unaffected. Consistently, IFNγ-induced STAT1-DNA binding ability was abrogated by SS. ECs after IFNγ treatment increased the expression of three STAT1-mediated CXC chemokines for T cells attachment, i.e., IFN-inducible protein-10 (IP-10), monokine induced by IFNγ (Mig) and IFN-inducible T-cell a-chemoattractant (I-TAC). SS to IFNγ-treated ECs

reduced the expression of these chemokines. Furthermore, the inhibitory effect of SS on the IFNγ-induced responses was not observed in those ECs subjected to disturbed flow. Our results provide a new insight for the athero-resistant nature in the straight segments of arterial tree where cytokine-induced responses including T cells recruitment are suppressed.

2880

**Effects of Hyaluronan on Inflammation-Induced Angiogenesis**

M. Onodera,[1,2] T. Muneta,[3] K. Miyazaki,[4] H. Yoshimasu,[1] I. Morita[2]; [1]Community Oral Health Care Science, Tokyo Medical and Dental University, Tokyo, Japan, [2]Cellular Physiological Chemistry, Tokyo Medical and Dental University, Tokyo, Japan, [3]Orthopedic Surgery, Tokyo Medical and Dental University, Tokyo, Japan, [4]Seikagaku Corporation, Tokyo, Japan

Hyaluronan (HA), a core component of extracellular matrix, plays multifaceted roles in patho-physiological conditions. Among a lot of functions, we focused on effects of HA on inflammation-induced angiogenesis. First, we examined the direct effect of different molecular size of HA on angiogenic activity in bovine endothelial cells (EC) in vitro. The angiogenic activity was assessed by tube forming activity of EC in type I collagen gel. EC cultured between collagen were morphologically changed and formed tube-like structure. HA (MW<200K) had no effect on angiogenic activity, but HA (950K) used clinically inhibited it in a dose dependent manner. The inhibitory effect of HA (950K) was also observed in angiogenic activity induced by VEGF, and the inhibition was partly abolished by an addition of neutralizing antibody against CD44, one of the HA receptors. Next, we examined the effect of HA on inflammation-induced angiogenesis. The angiogenesis in rheumatoid arthritis (RA) has a crucial role in the progression of the disease. Synovial cells were isolated from RA and osteoarthritis (OA) patients and cultured successively. The production of prostaglandin E2, one of the angiogenic factors, induced by IL-1 was dramatically inhibited by HA (950K) via inhibition of COX-2 expression in synovial cells. In synovial fluids, HA is decomposed to lower molecule HA by hyaluronidase. Therefore, finally we identified hyaluronidase in the synovial fluid. As a result, hyaluronidase in the synovial fluid was identified as hyaluronidase-1, and the activity in RA-synovial fluid was higher than that in OA-synovial fluid. (Conclusion) High molecule HA inhibited angiogenesis directly and indirectly in inflamed tissues such as RA, and endogenous hyaluronidase-1 may regulate the anti-angiogenic activity of HA.

2881

**Human Endothelial Cells Secrete ST2, an Interleukin-1 Receptor Family Member, in Response to Inflammatory/Growth Stimuli**

K. A. Kassik, E. O. Weinberg; Medicine/Cardiovascular, Boston University Medical Center, Boston, MA

ST2 is an interleukin-1 (IL-1) receptor family member encoding a membrane-anchored receptor and a soluble secreted receptor that directs T cell function and negatively modulates interleukin-1 receptor signaling in T cells and macrophages. We have previously shown that serum levels of soluble ST2 predict mortality in cardiovascular diseases in humans. We tested whether human endothelial cells can secrete soluble ST2 and studied signaling pathways involved in ST2 induction in cultured human fibroblasts and endothelial cells. Methods: Fibroblasts were treated with phorbol ester (PMA, 0.2 microM) with and without the ER/Golgi vesicular traffic inhibitors, brefeldin (5 microg/mL) or monensin (200 nM). Endothelial cells were treated with PMA, IL-1 beta (5 ng/mL), VEGF (100 ng/mL) or TNF-alpha (100 ng/mL). Culture media was collected and assayed for ST2 (pg/mL) by ELISA (N=3-6/group). Results: Fibroblasts secrete ST2 in response to PMA (4.2 fold, 0.053+0.005) vs. control (0.012+0.002, $p<0.01$). Brefeldin prevented PMA-induced ST2 secretion (1.2 fold vs. control, NS). Monensin paradoxically enhanced PMA-induced ST2 secretion (8.7 fold, 0.10+0.006, $p<0.001$). In endothelial cells, ST2 secretion was induced by PMA (2.7 fold, 0.36+0.003), interleukin-1beta (3.2 fold, 0.427+0.005), VEGF (1.5 fold, 0.201+0.006) vs. control (0.133+0.006, all $p<0.01$). TNF-alpha did not induce secretion of ST2. In endothelial cells, ST2 secretion was completely blocked by ERK inhibition (U0126, 10 uM or PD098059, 10 uM), 50% blocked with JNK inhibition (SP600125, 50 uM), and marginally blocked (8%) with p38 inhibition (SB203580, 15uM). Conclusions: Induced secretion of ST2 involves ER/Golgi trafficking (blocked by brefeldin). Off-target effects of monensin (Na ionophore) may be the mechanism for enhanced ST2 secretion. Endothelial cells secrete ST2 in response to growth/inflammatory stimuli through ERK and JNK activation and are a potential source of elevated serum ST2 levels in patients with cardiovascular diseases.

2882

**Studies on *O*-GlcNAc Modification of NF-κB p65 Subunit**

W. Yang, S. Park, D. Kim, H. Kim, J. Cho; Biology, Yonsei University, Seoul, Republic of Korea

NF-κB is a transcription factor and is important for expressing variable proteins related to inflammatory and immune responses. It is composed of a heterodimer of p65 and p50 subunits and is located in the cytoplasm by I-κB. When the nuclear localization signal is transduced, I-κB is degraded by ubiquitn-dependent manner and NF-κB is transported to nucleus. Functions of NF-κB is regulated by variable posttranslational modification like phosphylation, acetylation and ubiquitination. Also, NF-κB p65 is modified by *O*-GlcNAc but, the exact function of *O*-GlcNAc on NF-κB p65 has not been studied well. *O*-GlcNAc is dynamically modified in a β-linkage to serine or threonine residues of nuclear and cytoplasmic proteins by two enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase. In this report, roles and sites of *O*-GlcNAc modification on NF-κB p65 using human lung carcinoma cell, A549 has been investigated. We demonstrate that show that the treatment of STZ (streptozotocin), an *O*-GlcNAcase inhibitor, increases *O*-GlcNAc modification and the activity of NF-κB p65 without the degradation of I-κB and inhibits the binding of NF-κB p65 with I-κB. Also, we identified Thr 352 as a target for *O*-GlcNAc modification and transcriptional activation in response to STZ by mutational analysis. Moreover, *O*-GlcNAc modification and the transcriptional activity of NF-κB p65 were increased in STZ-induced diabetic mice. Our finding suggests that *O*-GlcNAc modification on Thr 352 of NF-κB p65 subunit increases the transcriptional activity and the increment of O-GlcNAc on NF-κB p65 subunit may be a reason for diabetic-associated NF-κB p65 subunit activation in diabetic mice. Therefore, our data may contribute to understanding of diabetes and its complications-associated NF-κB p65 activation.

2883

**Myocardial Ischemia-Reperfusion Injury Results in Less Cardiac Tissue Damage and No Serum IL-6 Concentrations in W/W$^v$ Mast Cell Deficient Mice as Compared to their Normal +/+ Littermates**

K. Farwell,[1] K. Bhattacharya,[2] M. Huang,[1] J. Cao,[1] D. Kempuraj,[1] J. Donelan,[1] T. Theoharides[3]; [1]Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA, [2]Department of Cardiac Surgery, University of Glasgow, Glasgow, United Kingdom,

[3]Pharmacology and Experimental Therapeutics, Biochemistry, and Internal Medicine, Tufts University School of Medicine, Boston, MA

Myocardial ischemia reperfusion (IR) injury complicates all forms of coronary artery revascularization. Cardiac mast cells have been implicated in IR; however, their involvement has never been quantified. We have shown that cardiovascular disease is associated with cardiac release of IL-6 and acute stress increases mast cell-dependent serum IL-6 levels in mice. In this study, we compared cardiac tissue susceptibility and serum IL-6 changes between the mast cell deficient ($W/W^v$) mice and their normal littermates (+/+). Twelve male $W/W^v$ mice and their +/+ littermates were anaesthetized with 2.5% isofluorane. The left coronary artery was ligated for 30 minutes. After 6 hours of reperfusion, the animals were sacrificed. The muscle viability was assessed on fresh whole-mount slices by the nitroblue tetrazolium histochemical assay and serum IL-6 concentrations measured with ELISA. Cardiac muscle viability was significantly higher in $W/W^v$ than the +/+ mice. Baseline serum IL-6 levels were higher in the +/+ controls (range=300-700 pg/ml, n=6) than $W/W^v$ mice (range=70-250 pg/ml, n=6) before IR and this level increased significantly after reperfusion only in the +/+ mice (range=500-800 pg/ml, n=6, $p<0.05$), while it remained the same in the $W/W^v$ mice (range=70-280 pg/ml, n=6). These results show that the absence of mast cells reduces the myocardial damage associated with IR injury. Furthermore, there is an attenuation is the inflammatory response, as measured by serum IL-6 levels, following this local insult. This finding entertains the prospect of developing prophylactic therapy - targeting selective inhibition of cardiac mast cell activation, in clinical situations involving medical or surgical myocardial revascularization (Supported by Theta Biomedical Consulting and Development Co., Inc., Brookline, MA; KF was a Tufts University Shader Family Summer Research Fellow).

2884

**Corticotropin-Releasing Hormone Induces Vascular Endothelial Growth Factor (VEGF) Release from Human Mast Cells via the cAMP/PKA/p38 MAPK Pathway**

J. Cao,[1] T. C. Theoharides[2]; [1]Departments of Biochemistry and Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA, [2]Departments of Biochemistry, Pharmacology and Experimental Therapeutics, and Internal Medicine, Tufts University School of Medicine, Boston, MA

Mast cells are involved in allergic and anaphylactic reactions, but also in innate immunity and inflammation. Corticotropin-releasing hormone (CRH), the key regulator of the hypothalamic-pituitary-adrenal axis responses to stress, also has proinflammatory peripheral effects, apparently through mast cells. We showed recently that CRH selectively stimulates human leukemic mast cells (HMC-1) and human umbilical cord blood-derived mast cells (hCBMCs) to release newly synthesized VEGF without the release of preformed or other newly synthesized cytokines. This effect was mediated through activation of CRH receptor-1 and adenylate cyclase with increased intracellular cAMP. However, the precise mechanism by which CRH induces VEGF secretion has not yet been defined. In this study, we investigated whether protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) signaling pathways are involved in this process. Using ELISA, we showed that CRH-induced VEGF release was dose-dependently inhibited by the specific PKA inhibitor H89 or the p38 MAPK inhibitor SB203580, but not by the specific inhibitors of MEK (PD98059), the upstream kinase of the extracellular signal-regulated protein kinase (ERK) or the c-Jun N-terminal kinase (JNK) inhibitor SP600125. Furthermore, CRH significantly increased PKA activity, which could be mimicked by the cell-permeable cAMP analogue 8-bromo-cAMP, and was blocked by H89 or the adenylate cyclase inhibitor SQ22536. Using Western blot analysis, we showed that CRH induced rapid phosphorylation of p38 MAPK, which was inhibited by H89 or SB203580. These results indicate that CRH induces VEGF release in human mast cells via the cAMP/PKA/p38 MAPK signaling pathways, thereby providing further insight into the molecular mechanism of how CRH affects the release of a key proinflammatory mediator. .

2885

**Surface-cleaning Effects of Macrophages on Titanium Particles**

Z. Xing,[1] L. Pillati,[1] C. Alley,[2] K. A. Hasty,[1] R. A. Smith[1]; [1]Orthopaedic Surgery, University of Tennessee-Campbell Clinic, Memphis, TN, [2]Smith & Nephew, Inc., Memphis, TN

Titanium wear debris can stimulate the secretion of macrophages and result in bone resorption around orthopaedic implants. Titanium particles are non-degradable in human body and their long-term effects in vivo are yet to be studied. Titanium particles (Johnson Matthey, 1-3μm) washed with 70% ethanol were labeled as “As received particles”. The particles that were further incubated in 0.78μg/ml Lipopolyssacharide for 24 hours were labeled as “LPS-bound particles”. Some particles were added into Macrophage (IC-21, ATCC) culturing flask. After 24 hours, the flask was flushed by PBS to remove the free particles. The macrophages with phagocytosed titanium particles were incubated for 48 more hours until lysed by distilled water. After lysis, the titanium particles released by the macrophages were collected and washed with water and labeled as “cell treated particles”. The particles were suspended in RPMI-1640 medium and the concentrations were determined by scanning electronic microscope. Different types of titanium particles were added in macrophage culturing plate with a particle to cell ratio of 300 to 1. The media were harvested after 18 hours and the levels of TNF-α in the media were measured by ELISA. As received particles stimulated TNF-α secretion to a level of 37.46±6.49pg/ml, while LPS-bound particles resulted in a TNF-α level of 284.66±32.25pg/ml in the medium ($p<0.01$). However, after treated by macrophages, the particles lost their stimulatory effects on TNF-α secretion (10.23±5.11pg/ml; and 15.11±8.28pg/ml for as received/cell treated and LPS-bound/cell treated particles, respectively), although they were still extensively phagocytosed by the macrophages. This indicates that the titanium particles phagocytosed by the macrophages undergo processing that alters their surface features. After death of the macrophages, the released particles can still be phagocytosed by other macrophages, however they will no longer activate the secretion of the macrophages.

2886

**Fluoride Regulates the Transcriptional Expression of MMP20 *in vitro***

Y. Zhang; Orofacial science, University of California, San Francisco, San Francisco, CA

Objective: Excessive fluoride intake during tooth enamel formation can result in enamel fluorosis, characterized by a delayed removal of amelogenin proteins as the enamel matures. In this study, we used ameloblast-lineage cells to investigate the effect of fluoride on the synthesis and secretion of matrix metalloproteinase 20 (MMP20), a proteinse involved in amelogenin hydroysis. Methods: Cultured ameloblast-lineage cells, isolated from human fetal tooth organs, were exposed to 10 μM fluoride and analyzed for synthesis of MMP20 by immunobloting and real-time PCR. Fluoride-specific effect on cell signaling pathways was investigated via gene array, enzyme activity blocking and Western Blot. Results:

Immunoblotting showed that 10uM NaF down-regulated the synthesis of MMP20 by 21.17%, but did not alter the amount of amelogenin and TIMP2 synthesized by the cells. Real-time PCR showed that 10uM NaF down-regulated MMP20 mRNA expression to 28.27% of the levels found in the non-treated cells. A human MAP kinase pathway gene array indicated that fluoride-mediated down-regulation of MMP20 transcription is related to the JNK/c-Jun MAP kinase signaling pathway. Meanwhile, Western blot analysis showed that fluoride suppressed the phosphorylation of JNK and c-Jun. Moreover, the JNK specific inhibitor SP600125 down-regulated the expression of MMP20, incidentally with the decline of phosphorylated JNK and c-Jun. DNA affinity precipitation assay showed that c-Jun bound to an AP-1 site on the MMP promoter. Conclusions: Those results suggest that the fluoride-mediated alternation of MMP20 synthesis is primarily regulated at the transcriptional level. A fluoride related reduction of MMP20 expression by ameloblasts through JNK/c-Jun signal pathway, may result in a disturbance of the balance between MMP20 and its protein substrates contributing to the mechanisms of enamel fluorosis.

2887

**Pathogenesis of Light Chain (AL) Amyloidogenesis**

S. Davern, C. Murphy, S. Bohne, J. Wall, A. Solomon; Human Immunology & Cancer Program, University of Tennessee Medical Center, Knoxville, TN

The mechanism(s) responsible for the pathologic deposition of monoclonal light chains (i.e., Bence Jones protein [BJP]) as fibrils in the organs or tissue of patients with primary (AL) amyloidosis is presently unknown. As part of an effort to elucidate the pathogenesis of this disorder we have utilized an *in vitro* experimental culture system in which BJPs obtained from individuals with and without amyloidosis were incubated for 1-3h with human or murine fibroblasts and the extent of interaction determined quantitatively using a highly sensitive fluoroimmunoassay and qualitatively by fluorescence microscopy. These studies revealed that amyloidogenic BJPs bound preferentially; notably, this reactivity markedly increased when these molecules were rendered less stable through chemical modifications. Based on these findings, we posit that *in vivo* exposure of amyloid-associated BJPs to local cellular-derived factors results in destablilizing tertiary structural alterations and ultimately their deposition as amyloid fibrils.

2888

**Cell Culture Model of Hemifacial Hyperplasia and PTEN/mTOR Regulation**

S. L. Yen,[1] Y. Kiyomi,[1] C. Eng,[2] C. Cheung,[1] S. Kuznetsov,[3] P. Robey[3]; [1]Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles,, CA, [2]Human Clinical Genetics Program, Ohio State University, Columbus, OH, [3]National Institute of Dental and Craniofacial Research, Bethesda, MD

The mechanisms involved in asymmetric facial overgrowth syndromes such as hemifacial hyperplasia(HFH) are not well understood. The PURPOSE of this study was to develop a model for comparing the difference between cells from normal and overgrowing bone. METHODS: Primary cultures of marrow stromal osteoblasts were developed from bone biopsies of normal and overgrowing bone. These cell cultures were seeded at the same concentrations and measured for cell size and number by flow cytometry. Affymetrix U95 microarrays were used to compare molecular phenotypes. The PTEN gene and promoter were sequenced; PTEN gene expression was examined by real-time PCR and Western blot analysis. Rapamycin(50 ng) was cultured with overgrown cell cultures for 72 hrs. RESULTS: Cell cultures from the overgrown side showed a 2-fold increase in cell size and cell number. A germline missense mutation was detected in the PTEN promoter which correlated with a 40% suppression of PTEN transcripts and protein. Akt-PKB phosphorylation increased in overgrown cells. Rapamycin treatment rescued the overgrown cell phenotype. CONCLUSION: The cell culture model suggests PTEN/mTOR regulation of bone cell growth in human patients with HFH

2889

**DALIS are Stress-Induced Protein Storage Compartments for Substrates of the Proteasome and Autophagy**

J. Szeto,[1] N. A. Kaniuk,[1] V. Canadien,[1] R. Nisman,[1] N. Mizushima,[2] T. Yoshimori,[3] D. P. Bazett-Jones,[1] J. H. Brumell[1]; [1]Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]Bioregulation and Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, [3]Cell Genetics, National Institute of Genetics, Mishima-Shizuoka, Japan

Dendritic cell aggresome-like induced structures (DALIS) are protein aggregates containing polyubiquitinated proteins. Originally identified in maturing dendritic cells, DALIS act as storage compartments for Defective Ribosomal Products (DRiPs) prior to their degradation by the proteasome. The purpose of this study was to investigate whether DALIS are able to form in other cell types. Here we demonstrate that DALIS can be observed in a variety of cell lines such as macrophages, fibroblasts, and epithelial cells in response to stress, including oxidative stress, transfection and starvation. In most cases, protein synthesis was essential for DALIS formation, indicating that DRiPs incorporation is required. Using immunofluorescence and electron spectroscopic imaging, we show that DALIS are protein-rich structures and are distinct from cytoplasmic stress granules, which can also form in response to a number of cellular stresses. Autophagy, which mediates the degradation of organelles, bacteria, and long-lived proteins, was found to regulate the DALIS phenotype. By using a chemical inhibitor of autophagy and an autophagy-deficient fibroblast cell line, we show that autophagy disruption enhanced DALIS formation under a variety of stress conditions. However, during starvation, DALIS formation in autophagy-deficient cells was only partially inhibited by protein synthesis inhibitors, indicating that both long-lived proteins and DRiPs can be targeted to DALIS. Our findings show DALIS formation can occur under a variety of stress conditions. Significantly, the DALIS phenotype is not exclusive to immune cells and can form in other cell types. Furthermore, we demonstrate that DALIS formation is negatively regulated by autophagy in response to stress, starvation, and infection. These studies indicate that in addition to DRiPs, long-lived proteins in the cytosol may also be incorporated into DALIS.

2890

**Inhibition of Retinal Photoreceptor Cell Apoptosis by αA Crystallin**

G. Wu, S. Saraswathy, N. A. Rao; Ophthalmology, Doheny Eye Institute, Los Angeles, CA

Previously in the early phase of experimentally-induced intraocular inflammation, commonly referred as experimental autoimmune uveitis (EAU), we found the nitration of cytochrome c (cyto c) and upregulation of αA crystallin in the retina. Nitrated cyto c is known to cause apoptosis, but this cell damage was not observed. In this study, we determine if αA crystallin prevents the induction of an apoptotic cascade initiated by nitrated cyto

c. S-antigen was used to induce EAU in Lewis rats. In this induction, the peak of disease is normally seen at day 14 postimmunization (pi). In retinas of day 5 pi, cyto c was nitrated by mitochondrial peroxynitrite and released into the cytosol. In Western blot, nitrated cyto c was co-immunoprecipitated with anti-αA crystallin, indicating a binding of αA crystallin to the nitrated cyto c. Further, an *in vitro* cell-free system was used to duplicate the features of *in vivo* apoptotic program. In this system, the addition of nitrated cyto c/dATP triggered the cleavage of procaspase-3, initially into an intermediate subunit p24, and then to the final executioner subunits p20/p17. In this assay, the addition of αA crystallin led to a dose-dependent accumulation of p24 subunit and eliminated the subsequent formation of the executioner p20/p17 subunits as revealed by the Western blot. When nitrated cyto c is released in day 5 pi, with apoptotic protease activating factor-1 and ATP, it activates procaspase-9. The active caspase-9 then initiates the activation of procaspase-3. In this mitochondrial pathway of apoptosis, our results indicated that αA crystallin binds to 1) the upstream apoptogenic molecule, nitrated cyto c and; 2) the proteolytically-cleaved intermediate caspase-3 subunit p24, thereby effectively curtailing the subsequent generation of active caspase-3 in the early phase of intraocular inflammation. Supported by NIH EY015714

2891

**Expression and Secretion of a Recombinant Protein Norrin in Yeast Cells**

B. S. Shastry,[1] R. Blackburn,[2] B. Kana,[2] N. Motwani[2]; [1]Biological Sciences, Oakland University, Rochester, MI, [2]ApoLife, Detroit, MI

The ocular manifestations of disorders such as familial exudative vitreoretinopathy, Norrie disease (ND), retinopathy of prematurity and Coats' disease are very similar and involve abnormal vascularization of the peripheral retina and retinal detachment. Previous studies have shown that all of these disorders are associated with mutations in the ND gene. In spite of this, little is known about norrin, its molecular mechanisms of action and its functional relationships with the detachment of the retina or the abnormal retinal vasculature. To obtain a large quantity of norrin for structural and functional studies, a cDNA fragment of approximately 450 bp was isolated by amplification of a plasmid containing norrin cDNA with a pair of primers. The primers contained an overlapping region of yeast secretory signal, XhoI and NotI restriction sites and a 6 x His - tag that was incorporated into the reverse primer. The amplified cDNA fragment was cloned into two yeast expression vectors containing either galactose or ethanol inducible hybrid promoters and transferred into yeast cells. A total of 12 clones were analyzed in four different shake flask fermentation for the presence of recombinant norrin using 4-20% Tris-Glycine gels under reducing conditions and GelCode Blue staining technique. Of the 12 clones analyzed, 5 clones were found to express a protein of approximate molecular weight 15kDa that was not detected in the control plasmid fermentation sample. This protein was secreted into the fermentation medium and not present in the cell extract of the fermentation sample. When attempts have been made to purify the protein using the Novagen His-Band system, no significant amount of purified protein was obtained.

2892

**Decreased Reproduction Capacity in Male Connective Tissue Growth Factor (CTGF/CCN2) Transgenic Mice**

Z. Zhou,[1] J. T. Hoskins,[1] S. Chakraborty,[1] D. R. Brigstock,[2] P. A. Harding[1]; [1]Zoology, Miami University, Oxford, OH, [2]Center for Cell and Vascular Biology, Columbus Children's Research Institute, Columbus, OH

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family. CCN family members are cysteine-rich proteins that are implicated in biological properties such as cellular proliferation, adhesion, extracellular matrix production, migration, and pathologies including tumor formation and fibrosis. An in vivo approach was taken to assess the biological significance of CTGF/CCN2 using transgenic mice. Transgenic mice were generated using the pIRES-EGFP expression vector using a bicistronic mRNA containing both human CTGF cDNA and enhanced green fluorescent protein (EGFP) coding sequences under the regulation of the cytomegalovirus immediate-early (CMV-IE) promoter. We observed a reduced reproductive ability for male CTGF transgenic mice. In order to begin to examine this phenomenon, male CTGF (n=6) and non-transgenic male littermates (n=6) were each placed with three wild-type female mice and plugs were examined every morning until bred. The ratio of plug to litter was 1:1 for wild-type males, whereas male CTGF mice exhibited a 4:1 ratio. Secondly, epididymal sperm was collected and examined for motility and number. No differences in sperm motility (~50% after 1 hr) or sperm number ($7.5 \times 10^5$ and $8.8 \times 10^5$ sperm/ml) between CTGF and non-transgenic littermates, respectively, were observed. However, upon histological examination of testes from 7-week CTGF and non-transgenic littermates using Masson-Trichrome, collagen was present within the interstitium of testes from CTGF transgenic mice. No collagen was present in the interstitium of male non-transgenic mice. Furthermore, electron microscopy (EM) of testes of 7-week CTGF transgenic mice presented occluded seminiferous tubules and an altered interstitium, as compared to non-transgenic littermates (n=4). In conclusion, we have generated a CTGF transgenic mouse model in which male CTGF mice have demonstrated a decreased reproductive ability that may be due to the formation of testicular fibrosis.

2893

**Investigation of Advanced Glycation End Products - Anterior Cruciate Ligament Transection Model in Rat**

S. Lee,[1,2,3] S. Choi,[4] G. Song,[4] C. Uhm[1,2,3]; [1]Department of Anatomy, Korea University College of Medicine, Seoul, Republic of Korea, [2]Brain Korea 21 Biomedical Sciences, Seoul, Republic of Korea, [3]Korea Lung Tissue Bank, Seoul, Republic of Korea, [4]Department of Rheumatology, Korea University College of Medicine, Seoul, Republic of Korea

Osteoarthritis (OA) is one of the most frequent diseases in human musculoskeletal system, and the incidence of OA is increased with age. Most characteristic change in OA is the structural change of collagen and loss of proteoglycan present between collagens. Advanced glycation end products (AGEs) are formed by the nonenzymatic glycation and increased in amount by aging and hyperglycemia. AGEs are usually accumulated in the long-lived protein such as collagen in the articular cartilage. Accumulated AGEs make articular cartilage more stiff and fragile. This study was designed to compare the structure of normal articular cartilage in the knee joint to that of OA induced by ACLT after AGEs induction by streptozotocin. Eighteen male Wistar rats (200g ~ 250g) were divided into 4 groups based on the treatment; AGE induction/ACLT, sodium citrate buffer treatment/ACLT, AGE induction/no ACLT and control (No AGE induction, no ACLT) group. AGE was induced by injecting 65 mg/kg of streptozotocin (Sigma) intraperitoneally. After 4 weeks of streptozotocin injection, ACLT was performed in ACLT groups. Weigh bearing tests were performed at 1, 2, 4, 7, 14, 21 and 28 days after ACLT in all 4 experimental groups. Rats were sacrificed, knee joints were dissected. AGEs and type II collagens in knee joint cartilages were visualized by immunochistochemistry and quantified by ELISA. Weigh bearing tests results were dramatically decreased after ACLT. Immunohistochemical staining for AGEs and type II collagen showed an increased labeling in AGE

induction/ACLT group. Measurement of AGEs and type II collagen in articular cartilages revealed the highest level of both in AGE induction/ACLT group. These results suggest the AGEs are associated with the developing OA. Continued research of AGE induction/ACLT would provide a basis for understanding of the pathogenesis of OA.

2894

**c-Jun N-terminal Kinase Programs Gene Expression Pattern toward Tissue Destruction in Abdominal Aortic Aneurysm**

H. Aoki,[1] K. Yoshimura,[1] Y. Ikeda,[1] K. Fujii,[1] A. Furutani,[2] Y. Hoshii,[3] N. Tanaka,[4] T. Ishihara,[3] K. Esato,[2] K. Hamano,[2] M. Matsuzaki[1]; [1]Molecular Cardiovascular Biology, Yamaguchi University School of Medicine, Ube, Japan, [2]First Department of Surgery, Yamaguchi University School of Medicine, Ube, Japan, [3]First Department of Pathology, Yamaguchi University School of Medicine, Ube, Japan, [4]Department of Radiology, Yamaguchi University School of Medicine, Ube, Japan

Abdominal aortic aneurysm (AAA) is a common disease among elderly people that is characterized by chronic inflammation of aortic walls with destructive remodeling of extracellular matrix (ECM). Because many patients are already at high risk of rupture at the point of initial diagnosis, a non-surgical therapy is much awaited to achieve the reduction of AAA diameter, a major predictor of its rupture. Here, we report that c-Jun N-terminal kinase (JNK) is the major determinant of the disease progression of AAA, which programs coordinated gene expression pattern toward the tissue destruction. JNK enhances the expression of matrix metalloproteinases, MMP-9 and MMP-2, in macrophages and vascular smooth muscle cells that mediate the tissue destruction in AAA. In addition, JNK suppresses biosynthetic enzymes for ECM in vascular smooth muscle cells. In vivo inhibition of JNK activity resulted in effective prevention of AAA development in the disease models in mice. In contrast, n vivo gene transfer of lysyl oxidase, an ECM biosynthetic enzyme, ameliorated the development of experimental AAA. Strikingly, pharmacological inhibition of JNK caused significant reduction of established AAA diameter with normalized tissue architecture. Taken together, our findings demonstrate that JNK represents a promising therapeutic target by which inhibition we can achieve regression of AAA.

2895

**Effects of Catechin Derivatives upon STAT1 Activity and Their Role on Inteferon-gamma-induced Intestinal $Na^+$-$K^+$-ATPase**

B. Igreja, P. Soares-da-Silva; Inst. Pharmacol. Ther., Faculty of Medicine, Porto, Portugal

This study evaluated the effects of a series of catechin analogues on interferon-γ (IFN-γ) induced STAT1 activation and tested effectiveness of active compounds in preventing IFN-γ-induced decreases in $Na^+$-$K^+$-ATPase activity. Treatment with IFN-γ markedly increased total and phospho-STAT1 in human intestinal epithelial Caco-2 cells. The abundance of total STAT1 and phospho-STAT1 was not affected by treatment of cells with (-)-catechin (C), (-)-catechin-3-gallate (CG), (-)-gallocatechin (GC), (-)-gallocatechin-3-gallate (GCG), (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) or EGC plus gallic acid (all at 20 μM) for 48 h. The IFN-γ-induced increase in phospho-STAT1 was completely prevented by pre-treatment of Caco-2 cells with EGCG (20 μM), whereas C, CG, GC, GCG, EC, ECG, EGC, and EGC plus gallic acid failed to prevent the IFN-γ-induced increase in phospho-STAT1. The increase in short circuit current ($I_{sc}$) by amphotericin B, a measure of $Na^+$-$K^+$-ATPase activity, was markedly attenuated in IFN-γ-treated cells. This effect of IFN-γ was completely prevented in cells that were treated for 48 h with EGCG, but not with EGC. Treatment of mice with IFN-γ resulted in a marked decrease in $Na^+$-$K^+$-ATPase activity in the proximal colonic mucosa, this being prevented by treatment with EGCG, but not by EGC. In conclusion, ECGC, which was unique among a series of catechin derivatives in effectively inhibit STAT1 phosphorylation and prevent IFN-γ-induced decreases in $Na^+$-$K^+$-ATPase activity, may be useful in a clinical setting in that IFN-γ-induced inhibition of intestinal $Na^+$-$K^+$-ATPase activity proceeds in parallel alterations in water and electrolyte handling at the intestinal level.

2896

**Experimental Ulcerative Colitis: Chemical and Bacterial Induction**

A. R. Jurjus,[1] I. Abdallah,[2] K. Barada,[1] R. Tohmé,[1] M. H. Mostapha,[3] S. M. Kamal,[3] J. Freud[4]; [1]Human Morphology, American University of Beirut, Beirut, Lebanon, [2]Human Morphology, American University of Beirut & Beirut Arab University, Beirut, Lebanon, [3]Beirut Arab University, Beirut, Lebanon, [4]INSERM, Strasbourg, France

Over the past 2 decades, more than 20 experimental models for ulcerative colitis have been developed supporting the concept that genetic, environmental and immunologic influences may all contribute to the disease process. The aim of this work is to develop and characterize an experimental model of chronic ulcerative colitis induced by chemical and bacterial factors. The experiment consisted of 132 adult Sprague-Dawley rats divided into 4 groups (G). G1 (Sham) consisted of 28 animals injected weekly with the vehicle 1% methylcellulose (MC) intrarectaly (IR). G2 consisted of 38 animals injected IR weekly with 100 μl iodoacetamide (IA) in 1% MC. On the otherhand, G3 consisted of 38 animals injected IR with IA and enteropathogenic E. coli independently every week. The last group G4 consisted of 28 animals injected IR only with enteropathogenic E. coli on a weekly basis. The experiment was stopped after a hundred days. Symptoms and signs like weight loss, diarrhea and bloody stools were recorded. In addition, the animals from each group were sacrificed on a weekly basis whereby gross abdominal inspection was performed for signs of inflammation (redness, vasodilatation…) as well as adhesions, necrosis and megacolon. Histological studies were done on biopsies from descending colon, jejunum and ileum. Preliminary results depicted the persistence of inflammation in the group G2 and to a higher degree in G3. However, weight loss was mostly in G3 in addition to persistent diarrhea with or without blood in the stools in this group compared to others. Furthermore, adhesions, redness and vasodilatation in addition to marked necrosis in the descending colon were also depicted in group G2, and, in particular, in group G3. Further characterization of the model is being carried out at the molecular, immunological and physiological levels. (Grant from The Lebanese CNRS).

2897

**Biogenesis of Lysosome-Related Organelles Complexes (BLOCs) in Human Genetic Disease**

A. Helip-Wooley,[1] W. Westbroek,[1] H. Dorward,[1] R. Boissy,[2] M. Huizing,[1] W. A. Gahl[1]; [1]NHGRI, NIH, Bethesda, MD, [2]Department of Dermatology, University of Cincinnati College of Medicine, Cincinnati, OH

Hermansky-Pudlak syndrome (HPS) is a disorder of impaired lysosome-related organelle (LRO) biogenesis. Defects in melanosomes in melanocytes and dense bodies in platelets are hallmarks of the disease and manifest clinically with oculocutaneous albinism and a bleeding

diathesis. There are seven subtypes of human HPS described. HPS-3, HPS-5 and HPS-6 generally have mild hypopigmentation with little or no bleeding, while HPS-1 and HPS-4 have severe albinism, bleeding and early death from pulmonary fibrosis. The correlation between disease subtype and severity is becoming more apparent as we learn how HPS proteins interact and function in Biogenesis of Lysosome-Related Organelles Complexes (BLOCs). HPS3, HPS5 and HPS6 proteins are found in BLOC-2. EM of melanocytes from patients with these subtypes reveals fewer mature melanosomes and an abundance of small tyrosinase (tyr)-positive vesicles mislocalized throughout the cell. We have found that HPS3 has a functional clathrin-binding domain and that HPS3 co-localizes with clathrin on small vesicles. We hypothesize that BLOC-2 binds these tyrosinase-positive vesicles and delivers them to early stage melanosomes. HPS1 and HPS4 are members of BLOC-3. The small mis-localized vesicles seen in BLOC-2 deficient cells are absent from BLOC-3 deficient melanocytes, but large, tyrosinase-containing multilamellar compartments are present. Additionally, some melanosomal and lysosomal proteins accumulate in the trans-Golgi region. It appears that BLOC-3 deficient cells fail to form the small vesicles that serve as transport intermediates for some melanosomal proteins. In contrast, BLOC-2 deficient cells form these vesicles, but they do not properly traffic to and/or fuse with maturing melanosomes. Thus, BLOC-3 acts at an earlier stage of LRO biogenesis, so HPS-1 and HPS-4 result in clinically more severe disease than BLOC-2 defects (HPS-3, -5 and -6). HPS illustrates a new paradigm of using cell biology to understand human genetic disease.

## Bioinformatics/Biological Computing (2898-2906)

2898

### Comparative Proteome Bioinformatics: Identification of Phosphotyrosine Signaling Proteins in the Unicellular Protozoan Ciliate *Tetrahymena*

S. Gammeltoft,[1] S. T. Christensen,[2] M. Joachimiak,[3] E. Hill,[3] C. Guerra,[4] P. Satir[4]; [1]Department of Clinical Biochemistry, Glostrup Hospital, Glostrup, Denmark, [2]Department of Biochemistry, August Krogh Institute, Copenhagen, Denmark, [3]Computational Genomics Group, Department of Plant and Microbial Biology, University of California Berkeley, Berkeley, CA, [4]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY

Protein tyrosine phosphorylation is characteristic of metazoans and a key element in intracellular signal transduction pathways of multicellular organisms. We used bioinformatics to identify protein tyrosine kinases (PTK) and phosphotyrosine signaling domains in the unicellular protozoan *Tetrahymena thermophila*. Hidden Markov models (HMM) searches of the *Tetrahymena* genome and available EST data were performed using http://hmmer.wustl.edu/ with HMM models for PTK and signaling domains. Eight proteins were found with catalytic domains similar to PTK and three have a predicted tyrosine phosphorylation site in the activation loop. We identified several tyrosine phosphorylation motifs for docking of SH2 domains found in metazoan adaptor proteins like Grb2, SH-PTP2, PLCγ, and Src. Based on the structural alignment of the catalytic domain, a large phylogeny tree was reconstructed showing that the *Tetrahymena* kinases are distinct from both canonical tyrosine and serine/threonine kinases. Structural modeling of the catalytic domain showed residues with identity to tyrosine as well as serine/threonine kinases. Finally, HMM searches gave a number of hits with similarity to protein domains involved in metazoan phosphotyrosine signaling including eight protein tyrosine phosphatases, one phosphoinositide 3'-kinase, one SH2, four SH3, seven calponin homology, and 24 pleckstrin homology domains. In conclusion, we have identified protein kinases in *Tetrahymena* that share features of tyrosine and serine/threonine kinases. The presence of proteins in metazoan phosphotyrosine signaling suggests that these *Tetrahymena* protein kinases may phosphorylate tyrosine. We propose that *Tetrahymena* PTK-like kinases are members of an ancient clade of protein kinases that may be classified as kinases with dual specificity possibly predating the serine/threonine and tyrosine kinase divergence.

2899

### Novel Methodology for Identification of Sumoylated Protein by Electrospray-quadrupole-time of Flight (ESI-q-TOF) Tandem Mass Spectrometry

J. Lee,[1] J. Jeong,[2] J. Seo,[2] S. Koh,[2] K. Lee[2]; [1]Ewha Womans University, Seoul, Republic of Korea, [2]Center for Cell Signaling Research, Division of Molecular Life Sciences and College of Pharmacy, Ewha Womans University, Seoul, Republic of Korea

Covalent attachment of small ubiquitin-like modifier (SUMO) represents the important post-translational modification that is involved in various biological processes including cell survival, apoptosis, and signaling processes. However, the regulatory mechanism of sumoylation is poorly understood because it is difficult to identify the sumoylation substrates *in vivo*. Here, we have developed the novel way to determine the sumoylation sites in protein substrates by using the SUMO mutant in amino acid residue 95. This mutant makes it possible to detect the sumoylation sites by nanoLC-ESI-q-TOF tandem mass spectrometry. Distinct type of peptides, sumoylation residue (GG) on lysine residue of substrate, is generated by trypsinolysis of sumoylated protein. This methodology is a novel way to determine the sumoylation site of protein, which will very useful to understand the biological functions of SUMO.

2900

### Protein Expression Profiling Using Gene Ontologies of Human Mesenchymal Stem Cells During Osteogenic Differentiation Induced by Ascorbic Acid-2-Phosphate, β-Glycerophosphate, and Dexamethasone

R. M. Salasznyk,[1] R. F. Klees,[1] A. M. Westcott,[2] S. Vandenberg,[3] K. Bennett,[2] G. E. Plopper[1]; [1]Biology, Rensselaer Polytechnic Institute, Troy, NY, [2]Mathematical Sciences, Rensselaer Polytechnic Institute, Troy, NY, [3]Computer Science, Siena College, Loudonville, NY

We applied 2D gel electrophoresis and 2D liquid chromatography tandem mass spectrometry (2D LC-MS/MS) to identify proteins that are expressed in human mesenchymal stem cells (hMSC) undergoing in vitro osteogenic differentiation induced by the osteogenic stimulants (OS) ascorbic acid-2-phosphate, β-glycerophosphate, and dexamethazone. To assess the effectiveness of OS in inducing osteogenic differentiation, and identify specific changes in protein expression which accompanied OS treatment, Progenesis workstation software and the Database for Annotation, Visualization and Integrated Discovery (DAVID) were used classify these proteins and compare them with those found in undifferentiated hMSC and fully differentiated human osteoblasts (hOST). Of the 534 spots identified in OS-treated hMSC 2D gels, 272 (64%) and 443 (78%) spots matched those found in hMSC and hOST gels, respectively. Of the 909 different proteins identified by 2D LC-MS/MS in all these cell populations, 144 (16%) were found only in hMSC; 154 (17%) were found only in OS-treated hMSC; and 121 (13%) were unique only to

hOST. Differential expression of some of the identified proteins was further confirmed by Western blot analyses. Significant differences in calcium-mediated signaling molecules and extracellular matrix components were found during osteogenic commitment and differentiation in OS-treated cells. Gene ontology analysis with DAVID revealed that these functionally related proteins may serve as novel biomarkers for identifying hMSC commitment along osteogenic fates and demonstrate that protein expression profiling is a valuable tool in elucidating the osteogenic differentiation of hMSC.

2901

**Dynamic Regulation of T Cell Receptor Signaling Pathway Activation in Response to Altered Peptide Ligands**

L. Wille,[1] M. Kemp,[2] C. Stokes,[3] L. Nicholson,[4] D. Lauffenburger[2]; [1]Biology, Massachusetts Institute of Technology, Cambridge, MA, [2]Division of Biologicial Engineering, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, [3]Entelos, Inc., Foster City, CA, [4]School of Medical Sciences, University of Bristol, Bristol, United Kingdom

T lymphocyte response to antigen is determined, at least in part, by the affinity of the T cell receptor (TCR) for the antigen ligand. We propose that qualitatively and quantitativly different signals are triggered by low and high affinity TCR ligands, for these signals induce distinct cell fates including the production of IL-2 and other cytokines. The capacity of T cells to recognize a set of altered peptide ligands (APLs) -- ligands with amino-acid substitutions within an antigenic peptide -- provides an opportunity for examining how subtle ligand changes can lead to different cellular responses. Our hypothesis is that multiple signaling pathways dynamically encode information in a combinatorial network state which can translate small changes in TCR ligation events into predictable differences in cellular outcomes. To test this hypothesis we are defining a quantitative, multidimensional map of TCR avidity, signaling events, and cytokine production/apoptosis. A mouse T cell hybridoma line 1B6 is activated with three APLs to define changes in signaling pathways' activation that lead to quantitative differences in activation-induced cell death and cytokine production. Cellular outputs as measured via ELISA and FACS demonstrate large changes in IL-2 and apoptosis between the different APLs. Intracellular signaling events for the same APL conditions are measured using semi-quantitative phosphoprotein western blots and high-throughput kinase activity assays. Our studies focus on the signaling dynamics of Erk, p38, Akt, JNK, MK2, NFAT, and IKK. Partial least squares regression is applied to extract statistical relationships between TCR ligation, signaling events, and cytokine production/apoptosis. To date, our methodology has provided unique signatures in the ERK, JNK, Akt, and p38/MK2 pathways associated with weak and strong APL avidity. Our statistical model can successfully account for IL-2 production associated with these activation patterns.

2902

**Modeling Molecular Interactions to Understand Spatial Crowding Effects in Heterodimer Formation**

K. M. Puskar,[1] A. Parisi-Amon,[2] S. Ta'asan,[3] R. Schwartz,[4] P. R. LeDuc[1]; [1]Mechanical Engineering, Carnegie Mellon University, Pittsburgh, PA, [2]Biomedical Engineering, Duke University, Durham, NC, [3]Mathematical Science, Carnegie Mellon University, Pittsburgh, PA, [4]Biological and Computer Science, Carnegie Mellon University, Pittsburgh, PA

Molecular crowding occurs in cells when the density of interacting molecules is sufficient to create deviations from traditional models of chemistry in diffusive systems. Including crowding in models is essential to accurately capture the kinetics of macromolecular complex formation within the cell. These formations are prevalent throughout biology, such as in protein-protein interactions and DNA hybridization. In order to model binding in a crowded environment, we use a lattice based Monte Carlo simulation with both inert, and interacting molecules that bind to form heterodimers. Inert molecules, which occupy space but are not actively involved in the reaction process, might move throughout the solute along with the reactant species by passive diffusion or might be anchored at fixed positions within the solute. While the inert molecules might be expected to exert similar excluded volume effects on reactant molecules under either assumption, they could produce differential effects on reaction progress through their different influences on reactant molecular diffusion. We examine the relative contributions of the two models to crowding effects by examining the effect of moving inert particles versus stationery inert particles on the reaction rates of heterodimer formation. The simulation results from these relatively simple interactions proved to be complex and distinct for the moving versus stationary inert particles. Stationary inert particles exhibited a bimodal response while moving inert particles had little effect on the simulated dimerization reactions. These results may have applications in fields such as cell biology, chemistry, and computational biology.

2903

**Using Classifier Learners to Distinguish between +/- Ras Cell Images**

A. N. Reed,[1] S. Chen,[2] G. Gordon,[2] R. Murphy[2]; [1]University of California, Irvine, Irvine, CA, [2]Carnegie Mellon University, Pittsburgh, PA

H-Ras is a cancer promoting gene that is present in 30% of cancers and has been known to distort the morphology of cells. This has proven to be a challenge for automated analysis of protein location since previous methods are influenced by cell morphology. The difference in morphology must be eliminated for proper unbiased analysis. In order to accomplish this, we extract texture features from a small region in the cytoplasm and use a logistic regression classifier to distinguish between Ras +/- images. NIH 3T3 cells, which were previously CD-tagged with GFP for a specific protein, were transfected with the Ras oncogene. The pairs of Ras +/- were compared to determine if there exist statistically significant differences. Of the eleven proteins analyzed, one demonstrated such a difference: Serum deprivation response protein. Visual inspection confirms that there is a difference between the textures of the Rse +/- images. Biological explanations through literature surveys are needed to support this experimental result. Sophisticated features and classification methods are also explored for investigating other significant differences between the pairs of images for future work. This research will in turn help biologists to better understand the purpose of the Ras protein.

2904

**Utilizing Biobase's Integrated Proteome, Transfac® and Transpath® Databases to Gain Biological Knowledge from Scientific Information**

K. L. Lennon-Hopkins, R. J. Johnson, F. J. Barkalow; Biobase Corporation, Beverly, MA

Genomics and proteomics, when combined with traditional research approaches, offer new opportunities to identify and characterize biological pathways, as well as comprehend changes in these pathways in disease. A challenge faced by researchers is to make sense of the wealth of scientific information generated by these approaches. The Biobase Knowledge Library (BKL) is an integrated information resource that allows researchers to access biological knowledge quickly and efficiently. The BKL comprises interconnected volumes that include the Proteome

databases, TransFac®, and TransPath® and organize public sequence information with information derived from comprehensive curation of scientific literature. Here, we illustrate the utility of the BKL as a tool for easily navigating the vast body of scientific information and for facilitating understanding that allows for efficient future experimental design. We sought to determine the connection between transcription factors associated with Diabetes and their downstream targets, in order to identify additional proteins that may be involved in Diabetes. Using the BKL Retriever search tool, we identified 322 human proteins associated with Diabetes. Of those, 265 displayed abnormal protein expression and 175 exhibited aberrant mRNA expression. Of the 322 Diabetes-associated proteins, 15 were transcription factors. We viewed relationships among these proteins in the interactive BKL Workspace and linked to a comprehensive HNF4A Proteome report that included Gene Ontology, expression, regulation, and protein modification information and links to cited references. Linking to TransFac and TransPath, we obtained details and interactive graphical displays of upstream regulators and downstream targets of HNF4A. We recognized HNF4A targets already linked to Diabetes, and we identified cytochrome P450 proteins as HNF4A targets that warrant examination for possible Diabetes associations. Complimentary BKL access for academic users is available for Disease Spotlight proteins, Public TransFac and Public TransPath (http://www.biobase.de).

2905

### A General Technique for the Segmentation of Individual Cells in Light Micrographs

Z. S. Pincus,[1,2] J. A. Theriot[2,3]; [1]Program in Biomedical Informatics, Stanford University School of Medicine, Stanford, CA, [2]Department of Biochemistry, Stanford University School of Medicine, Stanford, CA, [3]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

Many *ad hoc* methods exist for separating cells from background (and, with less success, from each other) in light micrographs. Here we present a general method for this task which is applicable to all cell types and imaging modalities. By using an explicit, parameterized "shape model" learned from training examples, our tool is free of the implicit assumptions about cell shape and packing which characterize *ad hoc* methods. The only assumption of the shape model is that cell shapes in a given population lie along quantifiable, stereotyped deviations from the population average (e.g. large vs. small, round vs. elongated, etc.). We free our method from assumptions about how pixel intensities relate to the cell-versus-background distinction by converting images into a canonical form. By using simple pixel-wise classification, we can convert many different image types (e.g. phase constrast, DIC, epifluorescence, etc.) into "likelihood maps" which represent the likelihood that a given pixel belongs to a cell or to the background. To find a single cell, we then iteratively fit the shape model's pose and shape parameters to the canonical image, and repeat the process to find all cells. Finally, we refine the initial fit by deforming the shape edges to fit the image within a level-set framework. This method has shown human-competitive results in separating out individual cells from the background and from each other on many different image and cell types, including fixed S2 cell cultures, growing bacterial mats, and membrane-stained *Drosophila* epithelia. Moreover, we have been able to use shape models to numerically parameterize the shapes of segmented crawling fish keratocytes and wild-type vs. mutant *Caulobacter*. This demonstrates the potential for truly general micrograph processing and quantitation methods identifying cells and describing variations in cell shape.

2906

### Protein-Arginyltransferases Are an Evolutionary Conserved Family of Enzymes Related to Bacterial Enzymes of Non-Ribosomal Peptide Synthesis

R. Rai,[1] A. Mushegian,[2] A. Kashina[1]; [1]Animal Biology Dept., University of Pennsylvania, Philadelphia, PA, [2]Stowers Institute for Medical Research and Department of Microbiology, Kansas University Medical Center, Kansas City, KS

Arginyltransferases (Ate1) are evolutionary conserved enzymes that mediate posttranslational N-terminal protein arginylation. Ate1 is nonessential in yeast, but has been recently found to be essential for cardiovascular development and angiogenesis in mammals. Mammalian Ate1 gene encodes four ATE1 forms that are produced by alternative splicing of homologous exons that are adjacent within the Ate1 gene and differ in substrate specificity, tissue expression, and intracellular localization. To study the evolutionary connections of arginyltransferases, we used methods of probabilistic sequence comparison and fold recognition and found that Ate1 is related to the FemABX family of tRNA-peptide transferases, which are involved in peptidoglycan biosynthesis in bacteria. Like FemABX, Ate1 appears to have evolved by duplication of the GNAT-like acetyltransferase fold and to contain a patch of negatively charged amino acids that is conserved only in a subset of GNAT acetyltransferases and may represent an oxoanyon hole directly participating in catalysis. We use site-directed mutagenesis to characterize the residues predicted to be involved in Ate1 catalytic activity, substrate binding, and specificity for different N-terminal residues.

## RNAi Technology (2907-2914)

2907

### Dual Fluorescence Assay for Target Validation of siRNA

S. Kojima, O. Danciu, S. Hammond, E. Brown, A. Mongiu, G. G. Borisy; Cell & Molecular Biology, Northwestern Univ. Med. Sch., Chicago, IL

Small interfering RNA (siRNA) is a crucial method for cell biology. An obstacle towards efficient application is that not all siRNAs are effective in gene silencing. As a result, selection of multiple target sequences and validation of knockdown are essential to identify effective sequences. We have developed a ratiometric assay system to evaluate knockdown effectiveness indirectly using two fluorescent reporters. Target sequences for test were embedded in the 3'UTR of the DsRed2 gene. Small hairpin RNA (shRNA) containing the knockdown sequence was expressed from a plasmid also expressing EGFP (Biotechniques 36:74-79 (2004)). Under optimized cotransfection conditions, the relationship between green and red fluorescence was linear, allowing quantitative fluorescence measurement at the individual cell level. Knockdown was assayed as the ratio of red to green fluorescence in each cell and was tabulated as population means with standard deviations. For a proof of principle, we tested six and nine target sequences of human vimentin and laminA/C, respectively. In both cases, the best target sequences identified by our ratiometric assay corresponded to the best target sequences as assayed by western blotting and immunofluorescence of endogenous protein. We applied our assay to identify effective siRNA target sequences of the mouse Arp3 gene. Transfection of shRNA expression plasmids for the two best targets caused similar morphologic changes to mouse melanoma B16F1 cells. Lamellipodia were greatly attenuated in agreement with the array-treadmilling model where dendritic nucleation from the Arp2/3 complex is a major source of lamellipodia protrusion. The results establish that our validation

assay is useful for identification of efficient siRNA target sequences. Since the image acquisition and analysis procedures are adaptable to image readers in an automated mode, our assay will facilitate high-content, high-throughput screening to identify effective target sequences. Supported by NIH GM64346.

2908

**A Modular, Tunable Mammalian Gene Switch Based on RNAi and Repressor Proteins**

T. L. Deans, C. R. Cantor, J. J. Collins; Biomedical Engineering, Boston University, Boston, MA

A number of technologies have been developed to control gene expression in eukaryotes. Much research has focused on the regulation of transcription using repressor proteins such as TetR and LacI, with only moderate success due to leakiness. More recently, RNA interference (RNAi) has advanced as a regulatory tool based on posttranscriptional gene silencing in which specific mRNAs are degraded. Despite these technologies offering ways to decrease gene function, neither method has proven to be an effective way to silence a gene. Here, we introduce an engineered tunable switch that couples repressor proteins and a novel RNAi target design to effectively turn any gene off. LacI is used for transcriptional repression of gene function and any leakage of the transcript is knocked down by the RNAi component of the switch. Gene expression is turned on by adding IPTG, which prevents LacI from binding in the Lac operator sites thereby allowing transcription of the gene. Additionally, IPTG turns the RNAi component of the switch off, allowing for the transcript to be retained and translated. To demonstrate its efficacy, the switch was used to regulate the expression of EGFP in CHO cells. In the absence of IPTG, these cells exhibit effectively no EGFP expression (>99% repression compared to positive controls). Furthermore, a dose-response increase of EGFP expression occurs with the addition of IPTG. These results indicate that the switch is not only capable of mediating on/off states, but it also allows for tunable expression of the gene. We show that the switch possesses reversible stringent control of gene expression, and that it is endowed with modular capabilities that allow for the regulation of any gene, as well as having the potential to be utilized in a tissue specific manner.

2909

**A Novel High-throughput Method for Electroporation of Adherent Primary Cells**

K. E. Jardemark; Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

To meet the demands of effective transfection methods for primary cells and stem cells, electroporation is developing into a powerful alternative to chemical/viral transfection methods, especially in the rapidly growing field of RNAi. Here we use a novel parallel method for electroporation of small cultures of adherent cells. Electroporation allows for introduction of any polar or charged agent that will not spontaneously pass the cell membrane. Addressing multiple adherent cultures in a parallel fashion not only enables perfect spatial control over affected cell areas, but also saves time, and offers real-time monitoring of treated cells. For example, it facilitates the identification and functional characterization of cytoplamic proteins. The technique is suitable for any type of adherent cells, especially cells considered difficult to transfect (*e.g.* primary cells, stem cells and neuronal cells) with agents of any type desired, such as drugs, oligonucleotides, siRNA, plasmids *etc*. Electroporation is performed by focusing capillary electrodes near the surface of adherent cells. The capillary electrodes are constructed to both deliver the transfection agent and an electric field locally to the cells. We have used the method for transfection of plasmids coding for various receptor/ion channel complexes, and silenced the expression of these complexes by transfection of siRNA. The yield and function of the expressed ion-channels, as well as the silencing efficiency of the siRNAs, were assessed by means of patch clamp and rtPCR detection. Moreover, the expression of GAPDH, as well as GFP, were silenced in primary endothelial cells and in a GFP-transfected endothelial cell line. The viability is generally high, i.e. over 90% and the efficiency for medium-sized molecules (*e.g.* siRNAs) is above 70%.

2910

**DNA Vector-based RNA Interference in *Spodoptera Frugiperda* Cells**

C. Lin; Life Sciences, National Tsing Hua University, Hsinchu, Taiwan Republic of China

Double-stranded RNA-mediated interference (RNAi) is rapidly emerging as a powerful tool used for the silencing of specific gene expression in many organisms. The dsRNA precursors might be processed into short interfering RNA (siRNA), which in turn mediates sequence-specific mRNA degradation. Herein, we describe a DNA vector-based method to achieve stable gene silencing in a *Spodoptera frugiperda* cell line, Sf9. With this approach, we have created a stably transfected Sf9 cell line, Sf9/pIBdsLuc, to express large dsRNA fragment mediating specific silencing of *luc*, the firefly luciferase gene. The *luc* dsRNA was constitutively transcribed from an inverted-repeat DNA sequence under the control of the OpIE2 promoter in Sf9/pIBdsLuc cells. Silencing of *luc* mRNA was observed when Sf9/pIBdsLuc cells were infected by rBacLuc, a recombinant baculovirus encoding *luc* gene. The luciferase activity upon rBacLuc infection, at the multiplicity of infection (MOI) of 1, in Sf9/pIBdsLuc cells was decreased by approximately 85% compared to that in control Sf9 cells. Thus, gene silencing in Sf9 cells could be effectively achieved using DNA vectors similar to the facile design described in this study.

2911

**Selective Purification of Small RNAs from Complex Biological Starting Materials**

C. Ritt, S. Carsten, R. Himmelreich, M. Weber; Chemical and Biological Research, QIAGEN GmbH, Hilden, Germany

In recent years, small RNAs have come more and more into the focus of research as they are ubiquitous, versatile molecules that play an important role in the regulation of gene expression and development in animals and in plants. Of special interest are miRNAs and siRNAs, small nucleic acids with a length of about 22 nucleotides. They are able to silence specific genes at the post-transcriptional level by virtue of their sequence complementarity to target mRNAs. To study the cellular role of these and other small RNAs in further detail, they must be isolated from tissue or cell culture in as pure a form as possible. Besides the removal of cellular components such as proteins, sugars, and lipids, nucleic acids that are longer than the desired RNA length should also be removed. We have developed a new method for the isolation of small RNAs from complex biological starting materials. The purification method is a simple and fast procedure that is based on magnetic particles. The method therefore offers convenient handling and is suitable for automation. Data will be presented that show efficient isolation of miRNAs with different combinations of buffers. Depending on the conditions selected, only nucleic acids in the size range of miRNAs are purified, while larger nucleic acids such as tRNAs are removed during the procedure. Varying the buffer conditions results in an isolation procedure that allows for the co-

purification of miRNAs and larger nucleic acids such as tRNAs or snRNAs. A further example will be shown that allows purification of virtually all size classes of nucleic acids contained in the biological starting material.

2912

**siRNA Libraries as Tools for Target Identification**

R. Jarvis, M. Byrom, D. Ovacharenko, C. D. Buchanan, D. Brown, L. Ford, D. Dorris; Ambion, Austin, TX

Small interfering RNAs (siRNAs) are routinely used to establish and confirm gene function in mammalian cells. We use libraries of siRNAs to identify genes involved in cellular pathways and processes and have measured how siRNA concentration, siRNA pooling, cell type, and time between transfection and analysis influence the final screening data. Our results provide a framework for setting up high throughput functional screens in both immortalized and primary cell lines with siRNA libraries and show the power of using such an approach to correlate genes with cellular functions. Using hundreds of siRNAs targeting different human kinases, we have discovered new roles for several of these kinases in apoptotic pathways.

2913

**Controlling Cell Signaling by Novel Transfection**

T. W. Sproul, A. M. Burt, L. T. Mazzola, R. D. Guettler; Excellin Life Sciences, Inc., Menlo Park, CA

Biological research has a compelling need to control processes within the cell, to turn on and off cellular functions through selective activation or inhibition of signal transduction pathways. The ability to transfer a wide range of exogenous materials into cells is of critical importance. Traditional transfection methods are primarily limited to nucleic acids and variously suffer from low efficiency, cell toxicity, and/or limited intracellular release. Here we demonstrate a novel microelectronic transfection approach that enables controlled delivery of virtually all biological components, including proteins, peptides, nucleic acids and small molecules. For comparison, we present data for siRNA into H460 cells, resulting in 90% apoptosis compared to 20% for lipid-based delivery of the same siRNA (5% observed control). We further demonstrate >95% induction of active fluorescently labeled antibodies and peptides into a variety of cell types. This novel process provides highly efficient induction into both suspension and *in situ* adherent eukaryotic cells with minimal impact to cell health - enabling unprecedented control over a wide range of cellular functions.

2914

**Verification of RNA Interference at the Protein Level, via Sequential Quantitative Proteomics Analysis**

J. J. Porter,[1] B. K. Ewing,[1] R. J. Mehigh,[1] S. L. Cockrill,[1] J. Wildsmith,[1] B. Boedeker,[2] A. Spencer,[2] R. Chiu,[2] M. C. Harvey,[1] J. G. Dapron,[1] K. M. Ray,[1] G. B. I. Scott[1]; [1]Proteomics R&D, Sigma-Aldrich Biotechnology, St. Louis, MO, [2]Molecular Biology R&D, Sigma-Aldrich Biotechnology, St. Louis, MO

The MISSION™ shRNA Library is a comprehensive collection of 150,000 pre-cloned lentiviral-based shRNA vector constructs targeting 30,000 annonated human (MISSION TRC-Hs 1.0) and mouse genes for RNA interference-mediated silencing. This lentiviral-based system allows for infection and integration into non-dividing and primary cells. RNA-mediated interference (RNAi) offers a mechanism for the study of gene function as well as target validation, but currently available methodologies to interrogate and measure protein levels following gene knockdown are inadequate and imprecise. Indeed, there is a pressing and demonstrable need to functionally validate mRNA knockdown by rapid and accurate quantitative surveillance of affected protein(s). Differential isotopic labeling of tryptic peptides using $^{18}O$ water is a well-established technique for measuring relative amounts of protein in two related but discrete samples. $^{18}O$ labeling is a global strategy in that essentially all peptides in a sample are labeled - so any changes between control and test samples are revealed. Absolute Quantification (AQUA™) represents a complementary quantitative proteomics technique to $^{18}O$ labeling. This method is a targeted strategy that exhibits robust efficacy and is being increasingly utilized for a wide variety of quantitative proteomics studies. Utilizing the MISSION shRNA Library lentiviral-based system, a variety of human kinases have been targeted for gene knockdown. Following gene knockdown, AQUA analysis of the targeted protein product and $^{18}O$ labeling are performed to observe both the target protein and other proteins potentially affected by the knockdown event. In the present work, we demonstrate that the $^{18}O$ labeling and PROTEIN-AQUA strategies can be sequentially coupled to RNAi technology in order to perform true quantitative verification of gene knockdown at the protein level.