Instituto Juan March
de Estudios e Investigaciones

148 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Membranes, Trafficking and Signalling during Animal Development

Organized by

K. Simons, M. Zerial and M. González-Gaitán

P. Bastiaens
S. Eaton
C. M. Field
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I. Guerrero
N. Hirokawa
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C. Martínez-A.
A. Martínez Arias
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Introduction

K. Simons, M. Zerial and M. González-Gaitán

One of the major questions of development is how equal cells in developing tissues differentiate into different cell types in a position-dependent manner. The generation of positional information involves a particular kind of secreted molecules, termed "morphogens", which emanate from a source, spread across the target fields and form long-range concentration gradients which provide the target cells with positional information. These receiving cells, in turn, interpret the gradient by activating target gene expression at discrete concentration thresholds. This simple model has been proven true during the development of many organs both during invertebrate and vertebrate morphogenesis. This includes dorsal/ventral patterning during early embryogenesis, limb development and neural and mesodermal patterning from flies to mammals.

Much of the success of the positional information model capitalized on the molecular identification of the distinct signaling molecules involved, many of which turned out to belong to the Wnt, Hedgehog, FGF and TGFβ families of secreted ligands. In addition, downstream these secreted ligands, the signal transduction pathways, including the receptors and mediators of the cellular responses, have been characterized in much detail at the molecular and biochemical levels. These data have provided a formal picture of signaling during morphogenesis: groups of cells send signals to target cells by generating gradients and the gradients are interpreted by means of rather linear signal transduction pathways which are initiated from a homogeneous plasma membrane.

During the past years a major effort has been taken to provide morphogenetic signaling with a novel cellular dimension beyond the formal molecular pathways and the mere description of inductive mechanisms. In particular, a very intense field of research addressed the role of membranes and their dynamic trafficking during i) the dispersion of the ligands across the target tissues which are thereby distributed as gradients of concentration and ii) the transduction of the signals which occurs not just at the plasma membrane, but at distinct membrane subcompartments within the developing cells. One example is the raft model, which proposes that different molecules mediating consecutive steps along signal transduction pathways cluster in lipid microdomains, rafts, which thereby control morphogenetic signaling. Other is the recent discovery that signal transduction may occur in some cases from an endosomal, intracellular compartment rather than from the plasma membrane. In addition, a picture is emerging whereby the dispersion of ligands may not always occur by free extracellular diffusion, but would involve membrane trafficking through the target cells.

The field is a very young and successful one and implies the mutual confluence of two scientific cultures: molecular developmental genetics and molecular cell biology. The meeting put together major players from both scientific communities that have contributed importantly in recent years to this novel interface research.

During the workshop we had the chance to discuss the molecular mechanisms underlying endocytic trafficking: from endocytosis to endosomal dynamics. We learnt about how a key GTPase Dynamin works to control the fission of endocytic vesicles (Schmidt) and, downstream this event, how small GTPases of the Rab family control the motility of endocytic vesicles and their fusion with endosomes (Zerial). A major focus in trafficking is what are the molecular mechanisms controlling the motility of organelles on

cytoskeletal elements: microtubules (Hirokawa) and actin (Small). The Rab proteins may play thereby a key role during development from flies (Gonzalez-Gaitan) to fish (Campos). During these trafficking events, phosphoinositides play a key role (Thorner).

Endocytic trafficking is at the core of morphogenesis, controlling developmental events from asymmetric cell division (Schweisguth) to morphogenetic signaling. Endocytosis can play different roles during morphogenetic signaling depending on the signaling event considered. It can control the trafficking pathway of receptors, determining its targetting towards degradation (TGF-beta signaling, Wrana). Endocytic trafficking can also control the spreading of ligands, either by promoting its planar transcytosis (Dpp, Gonzalez-Gaitan), perhaps vehicled by vesicular (argosomes, Eaton) or their degradation (Wingless, Vincent; FGF8, Brand; Hh, Guerrero). The control of the release of Hh ligands by sterol-sensing-domain proteins and trafficking was considered after the work of Ingham and Guerrero. Also, Aarole of endocytosis during the deposition of extracellular elements, such as the heparane sulfate proteoglycans was discussed (Perrimon) and its relevance during complex morphogenetic movements, such as the dorsal closure of the Drosophila embryo, is starting to be analysed in Martinez-Arias lab. During dorsal closure, Martinez-Arias discussed the role of the actin cytoskeleton and Wingless, a ligand that play a "catalytic" rather than instructive role.

In order to understand these events at the interphase between morphogenetic signaling and endocytic trafficking, it is key to have access to imaging technics that allow the visualization of signaling events with high temporal and spatial resolution. The work of Fraser and Bastiaen addressed this aspect. Fraser showed how sensitive imaging of espectrally overlapping fluorochromes by using a LSM META confocal microscope allows the simultaneous visualization of many molecules during development. He also proposed Fluorescence Cross-correlation as a powerful method to analyse protein-protein interactions. Bastiaen showed its very powerful and sophisticated FLIM analysis of signaling events and protein-protein interactions in vivo. Its work suggests a key role of peroxide species during the spreading of EGF-R signaling within a cell.

Finally we learnt the state of the art in the "raft" field. Simons presented the raft model, Parton showed how he could visualise the rafts at the EM level and Martinez-A. illustrated the role of rafts during polarization of migrating T-cells. In a challenging presentation, Kurzchalia told us about its analysis of the role of a key raft lipid, cholesterol, during development of the worm.

Kai Simons, Marino Zerial and Marcos González-Gaitán

Session 1: The endocytic pathway
Chair: Teymuras Kurzchalia

Rab domains in endosome organization and function

Zerial, M.

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Endocytosis is an essential activity of eukaryotic cells which serves many cellular functions, such as the maintenance of membranes homeostasis, the uptake of nutrients, intracellular signalling, the immune response and the defense against pathogens. Molecules internalised from the cell surface and transported to early endosomes from where they can be sorted to different intracellular destinations, including organelles of the degradative pathway (late endosomes and lysosomes).

To address the molecular mechanisms underlying endocytic transport we have focused on the function of Rab GTPases. Rab5 is a potent regulator of membrane dynamics. Rab5 regulates a molecular network of several effectors, each contributing a specific function in vesicle formation, tethering and fusion, as well as in microtubule-dependent motility of early endosomes. Importantly, Rab5 effector proteins function in a cooperative fashion. For example, the Rabaptin-5 /Rabex-5 complex activates Rab5 which in turn stimulates PI3-K activity. This lipid kinase regulates the recruitment of effectors binding both Rab5 and PI(3)P. Rab5 and its effectors are compartmentalized in a specific membrane domain and other endocytic Rab GTPases, Rab4 and Rab11, occupy distinct membrane domains on early endosomes that we call "Rab-domains".

Based on these observations we have formulated a new model to explain the organization and biogenesis of early endosomes. The model proposes that endosomes are organized as a mosaic of Rab-domains where each Rab-domain is created through the recruitment of effector proteins, which generate a spatially restricted and functionally specialized environment on the membrane. Divalent Rab effectors are responsible for the sequential communication between adjacent Rab-domains, for example from Rab5 to Rab4, from Rab4 to Rab11. Early endosomes can therefore be viewed as a modular system where each module or Rab-domain is formed by the self-assembly properties of lipids and proteins, has distinct biochemical composition and performs specific functions. A binary switch system (in the form of divalent Rab effectors) functionally links two contiguous Rab-domains thus allowing for the sequential transport of cargo along the entire endocytic/recycling pathway.

I will review these findings and discuss some recent data demonstrating a link between the trafficking and signalling pathways.

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A loss of function screen of zebrafish Rab proteins using morpholino anti-sense oligonucleotides

Isabel Campos, Emma Kenyon, Derek L. Stemple

Rabs (Ras proteins of rat brain) are small GTPases of the Ras family and constitute the largest subfamily of proteins within this group. Rabs are key regulators of vesicular transport in eukaryotic cells at many different levels in both the secretory and endocytic pathways. They seem to be involved in vesicular formation, targeting and docking and membrane remodelling and fusion. There are five times as many vertebrate Rabs as yeast homologues (Ypts, for yeast protein transport), which probably reflects the involvement of vertebrate Rabs in specialised cell function and in various stages of embryonic development. We decide to make use of the anti-sense morpholino oligonucleotide (MO) technology to systematically knock down zebrafish Rab proteins and assess the phenotypes of embryonic development in the absence of the targeted proteins. We have so far identified 44 zebrafish Rab proteins. Thirteen out of the seventeen MOs tested gave a phenotype in highly specific developmental processes, such as epiboly (zRab5a), vasculogenesis (zRab13 and zRab14) or melanocyte formation (zRab1b). With this approach we expect to unravel the predicted link between individual Rab proteins and specific developmental processes.

Evidence that dynamin•GTP is a key regulator of endocytosis

Sandra Schmid

Dynamin is an ~100 kD multidomain GTPase required for clathrin-mediated endocytosis. It self-assembles into rings and helical stacks of rings. Self-assembly stimulates dynamin's GTPase activity ~100-fold (Hinshaw, 2000; Sever et al., 2000a). This activity is mediated by its GTPase effector domain, or GED, which functions as an assembly-dependent GAP (Sever et al., 1999). Two models prevail for dynamin function in endocytosis (discussed in Sever et al., 2000a, Song and Schmid, 2003). One suggests that it serves as a mechanochemical enzyme such that GTP hydrolysis drives a concerted conformational change – a "powerstroke", akin to molecular motors – to constrict the assembled dynamin collar and drive membrane fission (Hinshaw and Schmid, 1995; Stowell et al., 1999). The second suggests that dynamin functions as a regulatory enzyme – like all other GTPase superfamily members – to control downstream effectors required for vesicle formation (Sever et al., 2000b; Sever et al., 1999). In this model, self-assembly activates dynamin's intrinsic GAP to switch off the GTPase and terminate signaling. To distinguish between these two models we sought to examine the role of the assembly-stimulated GTPase activity of dynamin: does it drive a 'powerstroke' or does it function to terminate interaction with downstream effectors? The Drosophila homologue of dynamin is encoded by the *shibire* gene. Several mutations have been identified in *shibire* that result in temperature-sensitive paralysis due to inhibition of endocytosis and synaptic vesicle recycling. A screen for suppressor mutations of the ts2 allele of *shibire* resulted in the isolation of several *su*ppressors of *shibire (sushi)* mutations, all of which were intra-allelic (Ramaswami et al., 1993). The ts2 allele corresponds to G146S in human dynamin and maps to the critical switch 2 region of the GTPase domain. Interestingly, two of the intra-allelic sushi mutations (designated KVS and Shy) mapped to residues within GED. We have characterized the enzymological defect in dyn(G146S) and of the double mutants. We find that the ts2 mutant is defective in GTP binding and that endocytosis is restored by second site mutations in GED that profoundly inhibit both basal and assembly-stimulated GTPase activity. These findings suggest that a 'powerstroke' driven by assembly-stimulated GTPase activity is not required for endocytosis and instead suggest that dynamin•GTP functions through downstream effectors to mediate endocytic vesicle formation.

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Membrane lipids and membrane-associated proteins in signaling and morphogenesis: Lessons from yeast

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The budding yeast, *Saccharomyces cerevisiae*, has provided a genetically tractable organism in which to investigate many of the fundamental principles that govern the role of the plasma membrane in the transduction of extracellular stimuli into intracellular responses. A general rule that has emerged is that the membrane itself provides a platform for recruitment and assembly of the protein complexes that mediate cell signaling. Membrane association enhances the rate of formation of signaling complexes by spatially localizing the components, restricting their degrees of freedom, and increasing their effective local concentration. Specificity in what is attracted to the membrane is provided by the nature of the lipids present in the plasma membrane, as well as by the proteins embedded in or associated with it. Furthermore, spatial and temporal modulation of the levels of these membrane constituents further dictates what protein complexes are formed and where they are assembled. In this presentation, I will describe current studies from my laboratory in three areas that illustrate these principles.

In *S. cerevisiae*, a small (346-residue) adaptor protein, Ste50, interacts constitutively with a mitogen-activated protein kinase kinase kinase (MAPKKK), Ste11. This association is mediated by a so-called "sterile alpha motif" or SAM domain found at the N-terminus of each protein. SAM-mediated binding of Ste50 to Ste11 is required for optimal response in three different MAPK signaling cascades. Absence of Ste50 severely compromises signaling in the filamentous growth response pathway and in the Ste11-dependent branch of the high-osmolarity-glycerol response (HOG) pathway. In contrast, absence of Ste50 causes only a modest reduction of signaling in the mating pheromone response pathway. We have foumd that a putative Ras-association (RA) domain located in the C-terminal region of Ste50 is also essential for filamentous growth and HOG pathway signaling. However, instead of interacting with Ras2, the C-terminal domain associates with another small GTPase, Cdc42. Strikingly, however, Ste50 associates equivalently well with either the GTP- or GDP-bound forms of Cdc42 and requires residues on the surface of Cdc42 different from those needed to recruit other proteins that bind differentially to the GTP-bound state, e.g. the Cdc42- and Rac-interactive (CRIB) domain found in protein kinases of the PAK family, such as yeast Ste20, Cla4, and Skm1. Since Cdc42 is always tightly bound to the plasma membrane via its prenylated C-terminal CAAX box and nearby basic residues that interact with the head groups of acid phospholipids, our findings suggest that the function of Ste50 requires that it be localized constitutively at the plasma membrane. Consistent with this conclusion, we find

that Ste50 is fully functional when its entire C-terminus (residues 219-346), including the RA domain (residues 235-327), is deleted and replaced by the C-terminal nine residues (KKSKKCAIL) of Cdc42, which is the minimal element in Cdc42 responsible for its plasma membrane-specific targeting. Hence, Ste50 should be considered a permanent subunit of Ste11 that serves to tether Ste11 non-covalently to the plasma membrane. In the filamentous growth and HOG signaling pathways, Ste20, the upstream activator of Ste11, only resides at the plasma membrane via binding of its CRIB domain to Cdc42-GTP, which also activates the enzyme. Thus, the function of Ste50 ensures that Ste20 efficiently encounters its substrate (Ste11) and only does so when Ste20 is in its activated state.

Proper morphogenesis and cytokinesis of budding yeast during mitosis requires five related septins (Cdc3, Cdc10, Cdc11, Cdc12, and Shs1/Sep7), which form a ring of filaments around the mother-bud neck. All five possess a GTP-binding domain and, except for Cdc10, a predicted C-terminal coiled-coil. We found that Cdc10 and Cdc12, purified from bacterial cells, are active GTPases. A mutant, Cdc12(S43V), that binds but cannot hydrolyze GTP *in vitro*, had no phenotype *in vivo*, whereas a mutant, Cdc12(T48N), that cannot bind GTP, fails to localize properly at the bud neck, and displays elongated buds and cytokinesis defects, especially at 37°C. The analogous mutant, Cdc10(S46N), has similar defects and, strikingly, a *cdc12(T48N) cdc10(S46N)* double mutant is inviable at 37°C. In synchronized cultures of the *cdc12(T48N) cdc10(S46N)* cells, septin complexes assemble at the incipient bud site in G1 even at the restrictive temperature, but are unable to form a ring at the neck in budded cells after S-phase, a phenotype displayed by cells lacking the PAK, Cla4. Absence of Cla4 makes *cdc12(T48N) cdc10(S46N)* cells inviable at all temperatures; conversely, overexpression of *CLA4* suppresses the ring-assembly defect of the *cdc12(T48N) cdc10(S46N)* cells at 37°C. *In vitro* Cla4, but not a kinase-dead Cla4 mutant, phosphorylates purified septins. Proximal to the GTP-binding domain, all septins possess a tract of basic residues that has been implicated in phosphoinositide binding. Indeed, septin rings are not maintained at the bud neck at the restrictive temperature in cells carrying temperature-sensitive mutations in the phosphatidylinositol (PtdIn) 4-kinase, Stt4, or the PtdIns4P 5-kinase, Mss4. Rings are maintained at the restrictive temperature in cells carrying temperature-sensitive mutations in the PtdIns 4-kinase, Pik1, suggesting that it is specificially the Stt4- and Mss4-dependent pool of PtdIns-4,5-P_2 (PIP_2) that promotes stability of the septin ring. Consistent with this conclusion, all yeast septins tested to date bind preferentially to PIP_2 *in vitro* over other phosphoinositides. Our results demonstrate that GTP binding by Cdc12 and Cdc10, Cla4-mediated phosphorylation, and association with PIP_2 are all necessary to achieve proper septin-ring assembly.

In animal cells, recruitment of Ptdns 3-kinase by growth factor receptors generates 3-phosphoinositides, which stimulate 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 phosphorylates and activates downstream protein kinases that elicit physiological responses, including PKB/c-Akt, p70 S6 kinase, PKCisoforms, and serum- and glucocorticoid-inducible kinase (SGK). We showed that the *PKH1* and *PKH2* genes of *S. cerevisiae* encode protein kinases that share an essential function and whose catalytic domains closely resemble those of human and *Drosophila* PDK1. In fact, expression of human PDK1

rescues the inviability of *pkh1Δ pkh2Δ* cells. Likewise, the yeast *YPK1* and *YKR2* genes encode protein kinases that share an essential function and have catalytic domains closely resembling SGK; inviability of *ypk1Δ ykr2Δ* cells is rescued by expression of rat SGK. Purified Pkh1 can activate purified mammalian SGK and PKBα *in vitro* by phosphorylating the same residue as PDK1 and activates purified yeast Ypk1 by phosphorylating the equivalent residue. Ypk1 phosphorylates a consensus sequence similar to that phosphorylated by SGK and PKBα. However, neither PDK1 nor Ypk1 require PtdIns-3,4,5-P_3 for activity, consistent with the absence of this phosphoinositide in *S. cerevisiae* a nd the lack of pleckstrin homology (PH) or phox homology (PX) domains in these yeast proteins. Thus, although Pkh1 and Pkh2 act like PDK1, and Ypk1 and Ykr2 like SGK, the upstream activator is not a phosphoinositide. Indeed, it has been shown that sphingosine (4-dehydro-sphinganine) also can stimulate PDK1. Correspondingly, *in vitro* Pkh1 and Pkh2 are stimulated by nanomolar concentrations of the sphingoid base found in yeast, phytosphingosine (4-hydroxy-sphinganine). Our recent studies demonstrate that Pkh1 preferentially activates Ypk1, and Pkh2 preferentially activates Ykr2, and that these cascades function in a novel pathway required for expression of genes necessary for cell wall integrity. There is compelling evidence in both yeast and mammalian cells that membrane microdomains enriched in sphingolipids and sterols, referred to as rafts, are involved in the biosynthetic delivery of certain proteins to the cell surface. In this regard, we found GFP-tagged Pkh1 and Pkh2 both localize primarily to prominent plasma membrane-associated puncta, quite distinct from actin patches. Formation of these punta is completely blocked by a mutation that prevents sphingolipid biosynthesis, and fully restored by addition of phytosphingosine to the medium, suggesting that the puncta represent microdomains enriched in sphingolipids. Since the amount of phytosphingosine generated by hydrolysis of phytoceramide will depend on the concentration of that sphingolipid in the plasma membrane, the Pkh1-Ypk1 and Pkh2-Ykr2 cascades could represent a feedback control mechanism whereby membrane growth via insertion of sphingolipid-enriched vesicles is monitored and coordinately coupled to the expression of genes required for appropriate expansion of the cell wall.

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Session 2: Signalling dynamics and cytoskeleton
Chair: Alfonso Martinez Arias

Imaging reaction networks in signalling and morphogenesis

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Reaction state of proteins are sampled by quantitative fluorescence imaging microscopy in intact cells, maintaining the connectivity and spatial organisation between all components. With this approach we want to probe the capabilities of reaction networks in early signal transduction and of reactions that regulate the microtubule cytoskeleton. We have adopted a recursive approach between optical detection of protein reaction dynamics in cells in response to spatially localised perturbations and mathematical modelling of the system, initially analysing a minimal set of components. The often non-intuitive general properties of the system that emerge from the theoretical analysis can be verified in the experimental setting of living cells. For example, the members of the receptor tyrosine kinase (RTK) family operate within a reaction network constituted from homotypic and heterotypic interactions between receptors, and the complementary intrinsic tyrosine kinase, and protein tyrosine phosphates (PTP) activities. To probe for the capabilities of multi-component RTK signalling units, we have analysed the spatial and temporal aspects of epidermal growth factor receptor (EGFR) activation by imaging its phosphorylation in intact cells. Our experiments indicated that a mechanism relying on reactive oxygen species mediated PTP inhibition coupled to RTK activation is responsible for signal amplification and spreading of phosphorylation to non-ligand bound EGFR receptors. To investigate the implications of a coupling between PTP and RTK activity we formulated a minimal reaction network that allows us to understand the non-intuitive properties of such a feedback system and to devise experiments to further test these. The existence of a bistable state in this type of reaction network explains an observed threshold response to growth factors, and how a high level of phosphorylated receptors can be maintained in plasma membrane regions that were not exposed to ligand.

In order to assess the morphogenetic principles that govern asymmetric microtubule growth, the tubulin binding state of the 17 kDa tubulin sequestering molecule stathmin/op18 was investigated in cells. The Xenopus stathmin releases tubulin upon its phosphorylation on serines 16, 25 and 38, increasing the free tubulin concentration thereby promoting microtubule polymerisation. The kinases that phosphorylate stathmin, or their upstream regulator molecules, are localised to specific macromolecular structures of the cell, such as the plasma membrane or chromosomes. It is therefore possible that a gradient of phosphorylated stathmin originates from these macromolecular structures, thereby creating a local environment in the cell that promotes microtubule formation. In order to image the tubulin binding state of stathmin in cells, a conformational sensor where fluorescence resonance energy transfer is monitored between CFP and YFP fused to the N and C-terminus of stathmin has been developed. Ratiometric confocal imaging of this construct expressed in

Xenopus epithelial cells showed gradients of phosphorylated stathmin at leading edges of cells and around chromatin in mitosis. These findings suggest that polarized microtubule shapes are generated by regulatory principles in which a local state of the cytoplasm promotes microtubule polymerisation.

Microtubule tip complexes meet substrate adhesion complexes to arrange polarity for directed locomotion

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Cell movement is driven by the regulated and polarised turnover of the actin cytoskeleton and of the adhesion complexes that link it to the extracellular matrix. For most cells, polarisation requires the engagement of microtubules, which exert their effect by mediating changes in the activity of the Rho GTPases. Evidence from live cell microscopy suggests that these changes are effected in a very localised fashion at sites of substrate adhesion, via specific microtubule targeting interactions. The guidance of microtubules into adhesion foci is stimulated by an increase in tension in the actin cytoskeleton, suggesting the involvement of a mechanosensor in the cross-talk between microtubules and actin filaments.Total internal reflection fluorescence microscopy reveals that the targeting interactions are very precise, in that the polymerising microtubule tips target focal adhesion sites within a vertical range of 50nm. A model is proposed in which microtubule adhesion site targeting serves to bring molecular complexes bound at the tips and along microtubules in close proximity with adhesion complexes, to promote adhesion disassembly and remodelling of the actin cytoskeleton. The possible involvement of microtubule tip proteins in this process will be discussed.

Unequal segregation of Neuralized, a regulator of Delta endocytosis, during asymmetric cell division

Roland Le borgne, François Schweisguth

Notch receptor signaling regulates binary cell fate decisions in Metazoan. In *Drosophila*, Notch regulates the pIIa/pIIb fate decision in the sensory bristle lineage. In this lineage, sensory organ precursor cells, or pI cells, divide asymmetrically along the anterior-posterior axis of the fly to generate a posterior pIIa cell and an anterior pIIb cell. The pIIa/pIIb fate decision is regulated by the unequal segregation of the endocytic protein Numb. In the dividing pI cell, Numb localizes asymmetrically at the anterior cortex and specifically segregates into the anterior daughter cell, where it antagonizes Notch signaling via regulated endocytosis, thereby specifying the pIIb fate. Thus, fate asymmetry appears to be generated by regulating Notch receptor endocytosis. Here, we report that differential regulation of the endocytosis of the Notch ligand Delta also contributes to fate asymmetry.

Neuralized is an evolutionarily conserved ubiquitin ligase required for Notch signaling. We show here that Neuralized is required to up-regulate endocytosis of Delta in the pIIb cell and to activate Notch in the pIIa cell. Specifically, we found that the pIIb cell contained 2.5 fold more Dl-positive endocytic (Hrs-positive) vesicles than the pIIa cell. This asymmetry in Delta endocytosis did not depend on Numb but required Neuralized activity. Neuralized localized asymmetrically in the dividing pI cell and unequally segregated into the pIIb cell. Thus, Neur and Numb are unequally inherited by the same pI daughter cell. We propose that Neur promotes ligand endocytosis in the pIIb cell, thereby activating Notch in its sister cell, pIIa, while Numb antagonizes Notch signal transduction in the pIIb cell. Thus, Neur and Numb act in parallel as complementary cell-fate determinants to bias Notch signaling.

Mechanism of intracellular transport and kinesin superfamily motor proteins, KIFS: structure, function, dynamics and diseases

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Cells transport and sort various proteins and lipids following synthesis as distinct kinds of membranous organelles and protein complexes to the correct destinations at appropriate velocities. The intracellular transports are fundamental for cell morphogenesis, functioning and survival not only in polarized cells such as neurons and epithelial cells but also in all other cells. To elucidate this mechanism we have identified and been characterizing kinesin superfamily proteins, KIFs, usig molecular cell biology, molecular genetics, biophysics, X-ray crystallography and cryoelectron microscopy. In this symposium I will focus on KIF1Bβ, KIF17, KIF5 and KIF3 and present our recent studies about their functions and diseases related to KIFs.

KIF1Bβ, KIF17 and KIF5 are deeply involved in neuronal functions. KIF1Bβ transports synaptic vesicle precursors and plays essential roles on neuronal function and survival. Molecular genetic study revealed that KIF1Bβ is a responsible gene of human hereditary neuropathy, Charcot-Marie-Tooth type 2A. KIF17 conveys vesicles containing NMDA type glutamate receptors, important for memory and learning, in dendrites through the interaction with scaffolding protein complex, mLin10, mLin2, mLin7, and NR2B subunit of NMDA receptors. Transgenic mice study showed that overexpression of KIF17 enhanced spatial and working memory in a series of behavioral tasks and up-reguated NR2B expression with the potential involvement of a transcriptional factor, the cAMP-dependent response element-binding protein (CREB) and increased phosphorylation of CREB. On the other hand, conventional kinesin (KIF5) transports AMPA type glutamate receptors in dendrites via KIF5 tail-Glutamate Receptor Interacting Protein 1 (GRIP1) - GluR2 interaction. Furthermore, our study revealed that GRIP1 plays a significant role to drive KIF5 to dendrites. KIF3 is abundant in neurons while it is also expressed in other types of cells ubiquitously. Our gene targeting studies demonstrated that KIF3 is essential for determination of left-right asymmetry of our body through formation of monocilia of node cells which rotate and generate leftward flow of extra embryonic fluid, nodal flow. This nodal flow could generate concentration gradient of putative secreted morphogen at the left side of node, which could turn on switches of a gene cascade, express them strictly at left side, and determine left-right asymmetry. Thus, KIFs play a number of significant roles not only on various cell functionings but also on fundamental developmental events such as left-right determination and on the higher brain functions such as memory and learning.

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Vesicular trafficking and cytokinesys

Christine Field

Cytokinesis, the process by which a cell divides into two daughters, requires the coordinated action of cytoskeleton and membrane systems. The cleavage furrow is a transient organizational state of the actin cytoskeleton that contracts to divide the cell in two. In some systems, the furrow is also is a site of local exocytosis to provide new plasma membrane for the daughter cells, and exocytosis is thought to play a role in the final stages of cytokinesis in all cells. (Bluemink and de Laat1973, Skop and White 2001, Shuster and Burgess 2002). The last few years have seen an explosion of interest in the role of membrane trafficking in cytokinesis. Recent progress includes identification of GTPases and other proteins that may direct vesicle trafficking during cytokinesis (O'Halloran 2000, Fontijn 2001, Schweitzer and D'Souza-Schorey 2002). Perhaps the most interesting question in cytokinesis research is how the cleavage furrow is positioned at the cell center, so as to bisect the separated chromosomes. Microtubules have long been know to provide this positional information, and clues are starting to emerge as to the molecular mechanisms by which they position of cytoskeletal components and local exocytosis (Danilchik1998, Murata-Hori and Wang 2002).

Our work has focussed on identifying cleavage furrow components and understanding how they interact together to promote furrow assembly and ingression. We are also interested in how the spatially restricted signal that tells the furrow where to assemble is delivered. Our study of furrow components has focused on two conserved proteins, anillin, a 120 kD protein originally isolated by F-actin affinity chromatography, and the septins, a family of GTP-binding proteins that assemble into heteromeric complexes and filaments. Recently we have also taken a small molecule approach, screening for new drugs that either block cytokinesis in whole cells, or target known furrow proteins, starting with myosin II.

Anillin localizes to the nucleus during interphase, the cortex during mitosis, and the cleavage furrow during cytokinesis. It shows the strongest cleavage furrow enrichment of any cytoskeleton-associated protein, and provides an excellent cytological marker for cytokinesis (Field and Alberts 1995). It is required for successful cytokinesis in animal cells. Inhibiting anillin function with mutations or antibodies does not prevent furrowing, but does slow it, and prevent completion of cytokinesis (Oegema 2000). EM analysis of cellularizing Drosophila embryos containing anillin mutations reveal membrane defects.

In vitro anillin binds to at least three essential, conserved cleavage furrow proteins actin, myosin II and septins (Kinoshita 2002 and references above). It also contains a PH domain that may target it to the plasma membrane. We recently showed that a population of anillin co-fractionates with vesicles from Xenopus egg extract, and we hope to leverage this observation to set up an *in vitro* system for reconstituting aspects of vesicle targeting during

cytokinesis. Given this biochemistry, we postulate that anillin plays an adapter role in cytokinesis, helping link different furrow components together.

Septins are a family of GTPases conserved from human to yeast. They have been shown to be required for cytokinesis in some (budding yeast, Drosophila and mammals), but not all systems (Reviewed in Field and Kellogg (1999) and Gladfelter (2001). We have focussed on the polymerization of septins, to determine if they constitute a novel cytoskeletal component in the cleavage furrow. We recently expressed a heteromeric septin complex in baculovirus, and reconstituted its ability to form rings by itself, and to bind to actin filaments when anillin is also added (supporting an adapter role for anillin)--- (Kinoshita 2002). This work points predominantly to a structural or cytoskeletal role of septins, and we hypothesize that anillin and septins together form a polymeric assembly that stabilizes cleavage furrows. Others have identified a possible role of septins in membrane trafficking (reviewed in Trimble 1999). They bind phospholipids (Zhang 1999), the exocyst complex(Hsu 1998), and syntaxin (Beites 1999), and may regulate exocytosis. Together with our work, these data lead us to speculate on a role of septins, and anillin, in coordinating the cytoskeletal and membrane biology of cytokinesis

Our lab is next door to the Institute of Chemistry and Cell Biology (ICCB), providing access to large libraries of chemical and equipment for screening them (http://iccb.med.harvard.edu). We believe that cytokinesis research will benefit from new small molecule tools, and have taken two approaches to finding them. We have screened for compounds that block cytokinesis, using automated microscopy of Drosophila cells. We found ~20 active compounds out of ~60,000 screened, and are proceeding to group them by effect on cells. A few target actin, but the others have unknown targets we will try to identify in the future. We have also screened for inhibitors of a protein known to be essential for cytokinesis, myosin II, and found blebbistatin, an inhibitor that blocks myosin ATPase and gliding motility. Blebbistatin is specific for myosin IIs over other myosins. It prevents contraction of the furrow, providing an arrest point that we can exploit to dissect the microtubule-dependent targeting mechanisms. By using other drugs in addition to blebbistatin, we have shown that myosin II and anillin target to the furrow by independent mechanisms. Myosin II targeting is inhibited by several kinase inhibitors, while anillin targeting is not. These data are consistent with the known role of rho-kinase in targeting myosin, and they suggest that anillin may target by a kinase-independent pathway. Straight AF and Mitchison TJ et al., submitted.

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Intracellular trafficking by Star regulates cleavage of the Drosophila EGF receptor ligand Spitz

Tzruya R., Schlesinger A., and Shilo B.

Signaling by the EGF receptor (DER) pathway in Drosophila controls a multitude of developmental choices. It is orchestrated primarily by processing of the TGF alpha ligand Spitz (Spi). The regulation on the processing of Spi relies primarily on intracellular localization of the protein and regulated trafficking along the secretory pathway. We found that although mSpi contains a signal peptide and a transmembrane domain the protein does not reach the plasma membrane, but remains instead in the ER, both in Drosophila embryos, adult tissues and S2 cells. When Spi is cleaved, Star and Rho cooperate sequentially to promote Spi processing, where Star is required first to transport mSpi from the ER to the compartment containing Rho, and Rho subsequently facilitates the cleavage and secretion.

Spi retention and trafficking take place in mammalian Cos7 cells as well, indicating that components of these processes are generally conserved. When the CopI complex, which is responsible for the Golgi to ER retrieval of proteins, was eliminated from S2 cells, Spi failed to be retained. A structure–function assay revealed that a construct of Spi lacking the intracellular domain was not retained in the ER, but predominantly localized to plaques, indicating that the anchoring retention signal of Spi resides at the intracellular portion of the ligand.

A third phase of regulation lies in the subcellular localization of the cleaved form of Spi. This regulatory process is directed at restricting the activity of Spi that is prematurely cleaved in the ER either by Rho or by other proteases. This assures that only Spi molecules that were cleaved in the proper compartment will be biologically active. Furthermore, we demonstrated that the constitutively secreted DER ligands Argos and Vein are not retained in this fashion, and that retention of sSpi is dependent on its intact EGF domain, suggesting the involvement of a specific mechanism. In contrast to the CopI-dependent fashion of the ER retention of mSpi, the retention of sSpi was found to be CopI-independent, further emphasizing that these phenomena represent different levels of regulation, using different cellular machineries.

The above findings provide a robust system to assay for perturbations in the retention of the Spi precursor and the cleaved ligand, and the study of Star-dependent ER-to-Golgi trafficking of Spi. We are using high throughput screens of RNA-interference technology, to screen for genes involved in Spi localization and trafficking.

Session 3: Trafficking and signalling I
Chair: Philip W. Ingham

Trafficking and the control of TGFß receptor activity

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Transforming Growth Factor-beta (TGFß) signals through a family of heteromeric transmembrane Ser/Thr kinase receptors to control growth and differentiation. The Smad signal transduction pathway is one important signalling mediator for this receptor class that is important for controlling transcriptional responses to TGFß family ligands. In addition Smads regulate the turnover of certain partner proteins by recruiting and activating the Smurf family of C2-WW-HECT domain ubiquitin ligases. Of particular interest the inhibitory Smads, Smad6 and Smad7 can recruit Smurf1 and Smurf2 to the receptor complex to induce their degradation via ubiquitin-dependent mechanisms. Endocytosis of cell surface receptors is an important regulatory event in TGFß signal transduction, but how endocytic pathways function to regulate TGFß signaling and receptor turnover is unknown. We have found that TGFß receptors internalize into distinct endocytic pathways that fulfill different functions in regulating Ser-Thr kinase receptor activity. Whereas internalization into the EEA1-positive endosome, is important for TGFß signaling an alternative pathway where Smad7-Smurf2 ae located is required for rapid receptor turnover. Thus, segregation of TGFß receptors into distinct endocytic compartments regulates Smad activation and receptor turnover.

Morphogenetic signaling and the endocytic pathway

Marcos González-Gaitán

Morphogenetic signals, such as the *Drosophila* TGFß-homolog Dpp, are secreted from a source and traffic throughout the target tissues. This way, they form concentration gradients which endow the receiving cells with positional information. We show that DPP stays only transiently at the extracellular matrix of the target tissue, implying that only short-range Dpp propagation can be explained by unhindered diffusion of Dpp in between cells. Instead, our results using mutants in dynamin and endocytic Rab proteins show that Dpp long-range propagation involves intracellular traffic of the ligand through the endocytic pathway. In particular, we showed that Rab5 and Rab7 determine the range of Dpp signaling throughout the tissue. Our data suggests Dpp propagation throughout the tissue occurs intracellularly via the endocytic pathway. We propose that the slope of the Dpp gradient, and therefore pattern and size of the wing, is determined at the receiving cells by the ratio between the Rab7-mediated degradation of Dpp and its Rab5-dependent recycling to be released and eventually signal at the next cells.

We also explored the possibility that signal transduction is initiated from an endosomal compartment. In particular, SARA is essential to initiate Activin-like signal transduction from an apical endosomal compartment. I will present our recent progress to uncover the role of an apical signaling endosome during Activin/Dpp signaling.

Endocytosis controls Fgf8-signaling during zebrafish embryogenesis

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Fgf8 acts as a key signaling molecule during induction, patterning and differentiation of several tissues in vertebrate embryogenesis, for instance during early CNS development, maintenance of mesoderm, and induction of inner ear and heart development. In spite of its importance as a signaling molecule, the cell biological mechanisms controlling spreading and efficacy of Fgf8 in developing tissues are poorly understood. Through genetic and cell biological studies in developing zebrafish embryos, we have begun to study how mechanisms controlling membrane traffic can determine how Fgf8 propagation through tissue leads to a downstream response. Our results suggest that endocytic trafficking regulated by small GTPases of the Rab family is important in controlling the signaling range and/or decision of cells to respond to Fgf8.

Imaging signaling in single cells and developing embryos

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The explosion of progress in the fields of cell biology, biochemistry, and molecular biology has offered unprecedented knowledge of the components involved embryonic development. The dramatic progress of these reductionistic approaches poses the challenge of integrating this knowledge into an understanding of developmental mechanics that pattern and construct the embryo. The classic publications in the field of experimental embryology illustrate the power of describing cell behavior (cf. lineages, movements) and perturbing the embryo to test hypotheses of the underlying mechanisms. Advanced imaging techniques offer an important stepping stone between these disparate approaches, permitting questions about cellular and molecular signaling events to be posed in the most relevant setting of the intact embryo.

Imaging offers insights into the context of cell interactions and signaling. For example, by sealing a Teflon membrane into the shell of a chicken egg, confocal laser scanning microscopy (CLSM) or two-photon laser scanning microscopy (TPLSM) can be used to follow neural crest cells (NCC's) as they migrates in the intact embryo. The neural crest is a transient cell population in the developing vertebrate embryo, originating from the dorsal neural tube to form a broad range of derivatives in the adult animal. Multiple optical sections can be assembled into 4-dimensional movies (3-D structure followed over time) and demonstrate cooperative as well as competitive interactions among the migrating NCC's; similar time series offer insight into interactions between the NCC's and their environment.

To validate this *in vivo* data, we have been developing a novel form of sectioning microscopy, in collaboration with Resolution Sciences Corporation. We fluorescently label the neural crest and surrounding tissues, then automatically section through the tissue at 1 micron increments, capturing a fluorescent image of the block face after each section. As we move through the embryo we trade physical sections for digital images. Because the block-face is not deformed by the sectioning, these images can be easily registered and computationally reconstructed to yield the full three dimensional volume of the tissue at subcellular resolution. This data can then be viewed volumetrically or re-sectioned to generate any desired two dimensional sections from arbitrary orientations, irrespective of the original plane of sectioning. We have been using this approach to map out the migratory pathways of the avian trunk neural crest, and to collect three dimensional protein localization information, using antibodies.

In conjunction with these volumetric studies, we are exploring the cell biology of neural crest migration guidance *in vitro*. Eph receptors and their ligands, the ephrins, have been implicated in neural crest migration guidance by several laboratories. To better assay the signal

transduction and mechanism of action of various potential guidance cues, we have developed approaches to present primary neural crest cell cultures with highly controlled molecular environments. In the first, we photolithographically immobilize proteins on glass coverslips, explant primary neural crest cell cultures onto these patterns, and then image the cytoskeletal and membrane dynamics within the cells as they encounter a boundary of a potential guidance molecule. While such approaches can be used to create arbitrary patterns of a potential cue, the cell under study still controls the time and place that the signal is encountered. In our second approach, a laser trap is used to force interactions between a cell and a potential cue held on the surface of a small bead. This controlled application of a cue demonstrates dramatically faster responses of the cell to the application of ephrins than previously proposed.

In vivo imaging of multiple labels should offer the ability to test the roles proposed from the time-lapse and fixed specimen imaging. GFP-color variants offer the possibility of following multiple molecular species in the same cell, and fluorescence resonance energy transfer (FRET) allows molecular interactions to be assayed as it takes place. Both of these approaches require the ability to uniquely identify the signal from each dye or fluorescent protein, but CLSM and especially TPLSM often fail to separate the signals. Multispectral approaches, in which the entire spectrum is taken of the emitted light from each pixel, offers a potential solution, as the spectral data can be decomposed into its component parts by simple mathematics. Fluorochromes as similar as GFP and fluorescein can be separated unambiguously, and even small amounts of FRET can be detected by our approach (now available as the Zeiss LSM-510 Meta). Examples of these imaging approaches and the integration of the data with other imaging modalities will be offered to demonstrate the successes and challenges of intravital analyses of cell signaling.

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The Wingless morphogen gradient is established by the cooperative action of Frizzled and Heparan Sulfate Proteoglycans receptors

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We have examined the respective contribution of Frizzled (Fz) proteins and heparan sulfate proteoglycans (HSPGs) in the establishment of the Wingless (Wg) morphogen gradient in the wing imaginal disc. Our analysis demonstrates that a HSPG-based mechanism is responsible for Wg movement, while the Fz receptors, in addition to transducing the signal, modulate the slope of the Wg gradient. Fz receptors regulate Wg spreading most likely by the degradation of Wg via receptor-mediated endocytosis. Finally, the spreading of Wg can simply be accounted for via extracellular diffusion of Wg, and not vesicular trafficking of Wg.

Wnt signalling and planar polarization of actin dynamics in *Drosophila*

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The process of dorsal closure of the *Drosophila* is a morphogenetic event which results in the generation of epidermal continuity on the dorsal side of the embryo which, for the most part of embryogenesis is covered by the amnioserosa, an epithelium which will not contribute to the final larval tissue. Dorsal closure requires concerted cell shape changes between the amnioserosa and the epidermis (1, 2). These movements are directional and result in the contraction of the amnioserosa and the dorsal stretching of the epidermal cells. In the final phases of the process the epidermal cells develop dynamic actin processes on their side of contact with the epidermis which are essential for the sealing of the continuous epidermal sheet.

Briefly, dorsal closure results from a sequence of events: elongation of epidermal cells in the dorsoventral axis, planar polarization of their cytoskeleton which manifests in the generation of Actin Nucleating Centers and eventually in the formation of a cable of F-actin and dynamic actin based processes on their dorsal most side, the leading edge (LE), contraction of the amnioserosa, zippering along the anteroposterior axis and, finally, sealing of the epidermal sheet at the dorsal midline associated with a loss of actin dynamics and planar polarization (3). These processes occur in sequence and have to be coordinated for the final proper shape of the organisms to be achieved. Thus, individual controls of the activity of the cytoskeleton are coordinated on a large scale, through cell cell communication systems. Consequently the major signalling pathways (TGFß, Hedgehog, EGF, Wnt and Notch) have been shown to play roles in the process. While some of these probably play specific roles in specific cell biological events (TGF, EGF) others, in particular Wnt, appear to be required for most or all of them.

We are examining the role of Wnt signalling in the process of dorsal closure. We have observed that Wingless, a Drosophila Wnt, is required for the elongation and polarization of the epidermal cells as well as for the correct activity of the cytoskeleton at the leading edge (3). Some of these effects are mediated by the classical Wnt/ß-catenin signalling (4) but others cannot be accounted by this signalling mode and suggest a more direct effect of Wingless on the activity of the cytoskeleton (3). Analysis of the role of Wingless signalling also highlight some features of the process, in particular some of the parameters of the interaction between the epidermis and the amnioserosa.

Our results indicate that Wingless does not act as a specific signal for particular events but rather as a catalyst for all of them. The notion and activity of a catalyst is important when

several processes have to be coordinated in space and time as it is the case during dorsal closure.

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Session 4: Trafficking and signalling II: Wingless and Hedgehog
Chair: Scott E. Fraser

Wingless and argosomes

Suzanne Eaton

Tissue growth and patterning are regulated by secreted morphogens. Morphogens are produced in spatially restricted organizer regions and develop a graded distribution in the surrounding tissue that is used to determine cell fate. Morphogens also control tissue growth by a mechanism that is less obviously dependent on their graded distribution. Our lab is interested in the cell biological mechanisms that control the distribution of and response to the Wingless morphogen in the *Drosophila* imaginal disc epithelium.

Wingless is produced in a narrow stripe of cells at the dorsal ventral compartment boundary of the wing imaginal disc. Despite its strong interaction with heparan sulfate proteoglycans, it is dispersed over many cell diameters through the surrounding tissue. To explain how a molecule with high membrane affinity might be released from producing tissue, we proposed that Wingless might be carried on membrane or lipidic particles, which we have called argosomes (Greco et al., 2001).

We first visualized argosomes in wing imaginal discs that expressed GFP targeted to the plasma membrane by GPI addition. In these discs, GFPgpi is found on the plasma membrane of the cells that express it, but is also found in endosomes throughout the non-GFPgpi-expressing regions of the disc. Similar behaviour is exhibited by HRPgpi, whereas other membrane associated proteins like CD8GFP remain restricted to the cells that make them. This suggests that protein sorting may regulate the composition of argosomes and that GPI may be an efficient argosome targeting signal. Wingless is found at a high frequency in the same endosomes that contain argosomes, raising the possibility that argosomes are a vehicle for Wingless movement.

What do argosomes consist of? One possibility is that they represent intact membrane exovesicles containing both cytoplasmic and extracellular membrane leaflets. Such vesicles might be formed by budding from the plasma membrane, or budding within multivesicular endosomes – the latter process being similar to that which gives rise to exosomes. Alternatively, argosomes might be more similar to lipidic particles like LDL. We are currently addressing these possibilities biochemically, and are examining the distribution of exosome and lipidic particle markers in imaginal discs.

Which endogenous gpi-linked proteins might normally be present on argosomes and what might their function be? Dally and Dally-like are gpi-linked heparan sulfate proteoglycans (glypicans) that are required for Wingless function. They have been proposed to act by trapping secreted Wingless at the cell surface and making it available to its receptors, Frizzled and Dfrizzled2. An alternative mode of action might involve the release of Dally or

Dally-like on argosomes. To investigate these possibilities we have constructed fluorescent fusion proteins of Dally and Dally-like and examined their localization in imaginal disc epithelia. Our data show that both Dally and Dally-like are present on the plasma membrane of producing cells, but that only Dally is released from the cells that produce it and is found in multivesicular endosomes in non-expressing tissue. These data suggest that Dally and Dally-like probably both have autonomous functions in promoting Wingless signaling, but that Dally may play additional, non-autonomous roles.

To ask whether gpi was necessary to target Dally to argosomes, we replaced its gpi-addition sequences with a CD2 transmembrane domain. Surprisingly, this novel fusion protein was also released from the cells that produced it. Clearly, Dally contains gpi-independent signals that specify its release.

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Trafficking of the Wingless signal in Drosophila

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We are interested in the mechanisms that regulate the secretion, transport, and degradation of Wingless within developing epithelia.

Degradation. We have shown that degradation of Wingless is regulated during Drosophila embryogenesis (Dubois et al, 2001). In Drosophila embryos, Wingless is expressed in segmentally repeated stripes. Starting around stage 11, the spread of the Wingless protein is reduced towards the posterior relative to the anterior. Using transgenic embryos expressing a HRP-Wingless fusion protein we have shown that Wingless is preferentially targeted to lysosomes in cells at the posterior. Lysosomal degradation has a dramatic impact on Wingless activity since interfering with it (genetically or with drugs) leads to ectopic signalling. Thus degradation of Wingless affect its distribution and range of action in the embryonic epidermis. Using clones of cells that are deficient in the *deep orange* gene, we found that same is true in imaginal disks. In the embryo, degradation is regulated spatially and temporally. We would like to identify the components that internalise Wingless and target it to lysosomes. Presumably, this is occuring by receptor-mediated endocytosis and we are currently trying to identify the receptor(s) involved. Candidate receptors include three members of the Frizzled family of 7 transmembrane receptors, the single pass transmembrane receptor Arrow (a homolog of vertebrate LRP6), and the two glypicans Dally and Dally-like. We are using genetics and cell culture approaches with S2 cells to assess which of the above candidate is required for internalisation and degradation of Wingless.

Secretion/release. We have shown that, in the embryo, a relatively small amount of Wingless is released by producing cells with a large majority being retained within the secretory pathway and at the cell surface (Pfeiffer et al, 2002). Such retention requires the activity of the proteoglycan-modifying enzyme *sugarless* and we are trying to identify which proteoglycans are involved and in what compartment they might associate with Wingless.

Transport. This is very much work in progress. Our approach is to use HRP fusion proteins to discover the subcellular localisation of the relevant components (including Wingless and its receptors) in wing imaginal disks. We are also designing experiments to test the role of the various receptors in Wingless transport.

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SSD proteins and Hedgehog signalling in animal development

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The *Drosophila dispatched* and *patched* genes encode two related multipass transmembrane proteins required for the reception and secretion respectively of the Hh signal. Both Ptc and Disp are characterised by a sterol sensing domain (SSD), a motif found in a growing family of proteins implicated in intracellular trafficking. These include SREBP cleavage activating protein (SCAP), the Niemann-Pick disease protein NPCI, and the *C.elegans* protein CHE14. We have previously shown that mutations in the SSD of Ptc abolish its ability to suppress the activity of Smo, a serpentine protein essential for the transduction of the Hh signal (1). The way in which the SSD functions is unclear, though recent studies of SCAP have implicated it in regulating the binding of this protein to an ER retention protein (2).

In searches of the ZF EST databases we have identified two genes related to *Drosophila disp*. We have inhibited the activity of both genes in the zebrafish embryo using antisense morpholino oligonncleotides. The phenotypes of the Disp1 morphant but not the Disp2 morphant are consistent with its being required for Hh signalling. We have identified a mutation predicted to cause premature termination of Disp1 translation N-terminal to the first transmembrane domain. Embryos homozygous for the mutation show a wild type response to unprocessed HhN but an attenuated response to cholesterol modified HhN (HhNp). Thus, as in *Drosophila*, zebrafish Disp1 is specifically required for the secretion of the lipid modified form of the signal.

Inactivation of zDisp2 also has severe phenotype effects, but these appear to be unrelated to Hh signalling. Notably, the SSD of zDisp2 lacks a GXXXD motif that is conserved amongst a sub-set of SSD containing proteins as well as in members of the prokaryotic RND (resistance-nodulation-division) permease superfamily. The presence of this motif - which has been implicated in proton binding in the RND proteins - in Ptc and Disp proteins, has led to the suggestion that they may similarly act as pumps (3). To investigate the functional significance of this motif we have analysed the effects of mutating it on the *in vivo* activity of Ptc in *Drosophila*.

Molecular analysis of the *open-brain* mutation in mouse has implicated the small GTPase Rab 23 in the negative regulation of Hh signalling (4), suggesting a possible role for Rab23 in the trafficking of Ptc and/or Smo. We have investigated this possibility further in *Drosophila* using transgenic and RNAi approaches.

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Role of patched and dispatched in the mechanism of Hedgehog signalling

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The Hedgehog (Hh) morphogenetic gradient controls multiple developmental patterning events in *Drosophila* and vertebrates. The understanding of the Hh pathway is crucial because its importance in embryonic development and also because its implication in human diseases (reviewed by Goodrich and Scott, 1998). The genes *dispatched* (*disp*) and *patched* (*ptc*) are involved in Hh release and Hh reception respectively (reviewed by Ingham and McMahon, 2001). Both genes encode proteins with twelve putative transmembrane domains and a sterol-sensing domain (SSD). The SSD domain was found in proteins involved in cholesterol homeostasis or cholesterol-mediated signalling (reviewed by Kuwabara and Labouesse, 2002).

In *Drosophila* imaginal discs, the posterior compartment cells produce Hh protein, which signals to the anterior compartment cells. We have studied the secretion of Hh by the posterior compartment cells of the wing imaginal disc and the role of Disp in this process. We have found that Hh is actively endocytosed by a dynamine-dependent mechanism in the Hh producing cells. In *disp* mutants, which do not secrete Hh, Hh is accumulated in the basolateral plasma membrane and no apical endocytic Hh vesicles are observed. Accordingly, ectopic expression of Disp in *disp*$^-$ cells eliminates Hh from the basolateral membrane. Moreover, we have found that Disp protein is located in the basolateral plasma membrane and in apical endocytic vesicles. Altogether these results suggest that Disp transports Hh from the basolateral to the apical membrane where Hh is secreted.

It is known that Ptc, the Hh receptor, restrains both Hh diffusion and Hh signalling in the anterior compartment cells (Chen and Struhl, 1996). We have studied how endocytosis regulates the concentration and signalling activity of Hh in the anterior compartment cells of the wing imaginal disc. We have observed that Ptc limits the Hh gradient by internalising Hh through endosomes in a dynamine-dependent manner and targeting it for lysosomal degradation. We have also found that the *ptc*^{14} mutant does not block Hh diffusion because it neither sequesters nor internalises Hh. However, the response of Ptc^{14} protein to Hh is normal indicating that Ptc-mediated internalisation is not required for Hh signalling. Furthermore, both in this mutant and in mutants that do not produce Ptc protein, Hh was still found in cytoplasmic vesicles in the Hh-receiving cells, which strongly suggests the existence of a second mechanism of Hh internalisation independent of Ptc.

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Session 5: Cholesterol, Rafts and Caveolae
Chair: Sandra L. Schmid

Lipid rafts in membrane trafficking and cell polarity

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We are studying the mechanism of raft clustering and sorting in MDCK cells and yeast. Lipid rafts are assemblies of sphingolipids and cholesterol in the exoplasmic leaflet of the fluid bilayer probably interacting with the underlying cytosolic leaflet. These assemblies function as platforms in membrane trafficking and signaling. A number of proteins specifically interact with rafts and these can be identified by biochemistry and mass spectrometry. One important property is the size of lipid rafts. Lipid rafts are small around 50 nanometers in diameter. The key characteristic of these small units is that they can be clustered by different means and this property is essential for their function. Our lab has been analyzing the clustering of rafts in several different cellular processes. We have analyzed apical sorting which involves clustering of rafts into apical transport containers. The apical surface is there enriched in lipid rafts. Our present data suggest that the apical membrane behaves completely differently than their plasma membranes. Here the rafts become continuous, forming one percolating phase. It is now the non-raft proteins that become confined to small ponds within the interconnected rafts. We are also studying the role of rafts in amyloid precursor protein (APP) processing. We have found that rafts play a decisive role in the generation of beta- amyloid from APP. During endocytosis rafts seem to coalesce to allow β-secretase to cleave APP.

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Investigation of the role of cholesterol in *C. elegans*

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Aim of our study was to elucidate the biological role of cholesterol in a multicellular organism. In particular we are interested in cholestero/glycosphingolipid-enriched microdomains in the plasma membrane of living cells. These specialized microdomains are envisaged as a lateral association of specific lipids and proteins in which cholesterol plays an organizing role. Data accumulated in recent years from experiments with cultured cells, demonstrated that the existence of such lipid microdomains is crucial for a number of cellular functions. A nematode *C. elegans* provides excellent experimental model for such studies, because worms do not possess enzymes for *de novo* sterol biosynthesis and completely depend on dietary sterols.

As a first step towards reaching our goal, we have studied the distribution of sterol and mechanism of its transport in *C. elegans*. Using fluorescent analog of cholesterol – dehydroergosterol, which we applied to the animal by feeding, we detected its accumulation primarily in the pharynx, nerve ring, excretory gland cell and the apical membrane of gut of L1 – L3larvae. Later the bulk of dehydroergosterol accumulates in oocytes and spermatozoa. Males display exceptionally strong labeling of spermatids which suggest possible role of cholesterol in sperm development or fertilization. In the complementary approach we used a photoactivable cholesterol analog, photcholesterol, to identify the cholesterol binding proteins in *C. elegans*. Three major and several minor proteins were found specifically cross-linked photocholesterol after UV irradiation. The major proteins were identified as vitellogenins. *rme-2* mutants, which lack the vitellogenin receptor, fail to accumulate dehydroergosterol in oocytes and embryos and instead accumulated dehydroergosterol in the body cavity along with vitellogenin. Thus uptake of cholesterol by C. elegans oocytes occurs via an endocytotic pathway involving yolk proteins.

To directly assess the role of sterols in worms we developed very stringent conditions for the deprivation of them in the diet. This led to 100% penetrant arrest of worms in the second generation at L2 larval stage. The arrest is reversible and can be rescued by very low amounts of cholesterol. Despite minute quantities of cholesterol, worm membrane contains lipid microdomains. These results speak for cholesterol playing a signaling role in nematodes rather than to be involved in the structural organization of membranes. We also investigated different compounds that could substitute for cholesterol and in the mean time are trying to identify long-searched hormone of nematodes.

Caveolin-dependent internalization of plasma membrane Rac binding sites is regulated by integrins

Miguel A. del Pozo, Richard G.W. Anderson and Martin A. Schwartz

Activation of Rac1 induces its translocation to the plasma membrane, an essential step for activation of effectors (1). Previous studies have shown that Rac1 recruitment to the plasma membrane requires integrin-mediated adhesion (2,3) and that activated Rac1 is enriched in cholesterol-rich raft domains (4). We now show that loss of integrin-mediated cell adhesion causes a coordinate loss of the lipid raft marker GM1 and caveolae from the cell surface, accumulation in an intracellular compartment and inhibition of Rac1 recruitment to the plasma membrane. Active Rac1 binds preferentially to low density, cholesterol-rich membranes, and the binding sites are provided at least in part by lipids. Preventing GM1 internalization in non-adherent cells retains Rac1 targeting and effector activation. Caveolin-1 expression is required for internalization of GM1 and loss of Rac1 targeting in non-adherent cells. Microinjection of the caveolin-1 scaffolding domain peptide stimulates GM1 internalization and blocks Rac1 recruitment to the membrane. These results show that integrin-dependent recruitment of Rac1 to the plasma membrane is mediated by a caveolin-dependent membrane trafficking pathway.

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Rafts and signaling in the immune system

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Migrating cells must integrate spatial and temporal information provided by environmental cues; they do this by acquiring a polarized morphology that requires initially identical cell regions to adopt distinct properties so they can carry out specialized functions. Membrane rafts have been characterized as cholesterol- and glycosphingolipid-enriched domains that can be isolated as detergent-resistant membranes (DRM) on the basis of their resistance to extraction by cold non-ionic detergents. A role is proposed for rafts in cell migration, based on the observation that disruption of their integrity by depleting plasma membrane cholesterol inhibits cell polarization and migration.

Two distinct raft subtypes, based on ganglioside composition, have been identified at each cell pole in T cells. Leading edge rafts (L-rafts) are enriched in GM3, whereas uropod rafts (U-rafts) are GM1-enriched. Chemoattractant receptors of the chemokine family are reported to partition to and signal in L-raft domains and redistribute during chemoptactic responses; L-raft redistribution leads to chemoattractant receptor accumulation at the leading cell edge. Conversely, U-raft redistribution participates in the accumulation of cell adhesion receptors at the uropod during T cell migration. I will discuus the results obtained using time-lapse confocal microscopy to analyze dynamic redistribution of raft domains in chemoattractant-stimulated leukocytes. Specifically, the implications of PI3K and PTEN redistribution after chemoattractant stimulation.

Caveolae, lipid rafts, and Ras signaling

Robert G. Parton

Caveolae, surface pits enriched in caveolins, have been implicated in signal transduction and lipid regulation. A truncation mutant of caveolin, CavDGV, potently inhibited Raf activation by H-ras but not by K-ras suggesting that different Ras isoforms reside in distinct surface domains. The CavDGV mutant accumulated intracellularly on lipid bodies, inhibited their motility, and perturbed lipid regulation. Inhibition of H-ras signalling by CavDGV was reversed by co-expression of wild type caveolin or addition of cholesterol suggesting that CavDGV acts as a dominant negative mutant to perturb plasma membrane cholesterol in lipid raft domains required for H-ras function. A naturally occurring caveolin-3 point mutant which has been linked to limb girdle muscular dystrophy, Cav3-C71W, also inhibited Raf activation by H-ras but not K-ras. Inhibition was rescued by cholesterol addition suggesting that the Cav3-C71W mutant also perturbs cholesterol-rich raft domains.

Using novel electron microscopic methods we have analysed these surface raft domains at the ultrastructural level. Immunogold electron microscopy of plasma membrane sheets coupled with spatial point pattern analysis enabled us to characterize morphologically featureless microdomains, including lipid rafts, in situ and at high resolution. An inner plasma membrane lipid raft marker displayed cholesterol-dependent clustering in microdomains with a mean diameter of 44nm which occupy 35% of the cell surface. Cross-linking an outer leaflet raft protein resulted in the redistribution of inner leaflet rafts but they retained their modular structure. Analysis of Ras microlocalization showed that inactive H-ras is distributed between lipid rafts and a cholesterol-independent microdomain. Conversely, activated H-ras and K-ras occupied predominantly non-overlapping, cholesterol-independent microdomains. These results illustrate that the inner plasma membrane comprises a complex mosaic of discrete microdomains. Differential spatial localization within this framework could account for the distinct signal outputs from the highly homologous Ras proteins.

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POSTERS

Membrane microlocalization dictates specificities on the activation of H-Ras, K-Ras and N-Ras and the function of the exchange factor Ras-GRF 1/2

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Ras GTPases include the isoforms H-Ras, K-Ras and N-Ras. Despite their great biochemical and biological similarities, evidence is mounting suggesting that Ras proteins may not be functionally redundant. A widespread strategy for studying small GTPases is the utilization of dominant inhibitory mutants that specifically block the activation of their respective wild-type proteins. As such, H-Ras N17 has proved to be extremely valuable as a tool to probe Ras functions. However, a comparative study on the inhibitory specificities of H-, K- and N-Ras N17 mutants has not been approached thus far. Herein, we demonstrate that H-, K- and N-Ras N17 mutants exhibit markedly distinct inhibitory effects towards H-, K-, and N-Ras. H-Ras N17 can effectively inhibit the activation of all three isoforms. K-Ras N17 completely blocks the activation of K-Ras and is only slightly inhibitory on H-Ras. And N-Ras N17 can mainly inhibit N-Ras activation. In light of the recent data on the compartmentalization of H-Ras and K-Ras in the plasma membrane, here we present for the first time a description of N-Ras cellular microlocalization. Overall, our results on Ras N17 mutants specificities exhibit a marked correlation with the localization of the Ras isoforms to distinct membrane microdomains.

In this same context, we also present evidence that the Ras exchange factors Ras-GRF 1-2 and SOS differentially activate H-Ras in a fashion dependent on the subcellular compartmentalization. We show that Ras-GRF1/2 and SOS colocalize with H-Ras in the plasma membrane and the endoplasmic reticulum but not in the golgi. By specifically tethering H-Ras to these distinct locations we can monitor H-Ras activation by the different GEFs. By this method, we show that the DH and IQ domains of Ras-GRF 1/2 play an important role in regulating the activation of H-Ras in the different locations. Likewise, the inhibitory effects that Cdc42 displays on the activation of Ras by Ras-GRF, that we have previously reported, are also dependent on microlocalization. Overall, our results point to the subcellular compartmentaliztion of H-Ras as a key determinant of its activation and subsequent functions.

FGFs and FGF-signalling-antagonists are important for the dorso-ventral patterning of the early zebrafish embryo

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in collaboration with Frank Reifers and Michael Brand (for the study of sprouty4)

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A Bone Morphogenetic Protein (BMP) activity gradient specifies dorso-ventral cell identity in both invertebrate and vertebrate embryos. In the zebrafish, genetic studies have shown that the ventralizing agents BMP2b and BMP7 and their antagonist Chordino are required for the establishment of the dorso-ventral axis. The inactivation of *chordino* causes however only a moderate ventralization, suggesting that additional factors must restrict BMP activity.

We have shown that zebrafish FGF8 is expressed along a dorso-ventral gradient at the gastrula margin, suggesting its implication in dorso-ventral patterning. Accordingly, FGF8 overexpression causes an expansion of dorso-lateral mesodermal and neurectodermal derivatives at the expanse of ventral territories. FGF3 and FGF18 display similar dorsalizing activities. Further analysis showed that FGFs exert their dorsalizing effect through a repression of BMP expression. Importantly, FGFs affect dorso-ventral patterning already at the blastula stage, earlier than the BMP-binding BMP-antagonist Chordino. In accordance with a requirement of FGF signalling for the restriction of BMP expression, injection of a dominant-negative variant of zebrafish FGF-Receptor1 causes a massive expansion of the ventral BMP2b expression domain and a concomitant enlargement of the epidermis at the expanse of the neural plate.

The use of an ongoing large scale *in situ* hybridization screen has allowed us to show that FGF8 is the founding member of a novel synexpression group. This group comprises several FGFs (3,4, 17,18), potential transcriptional mediators of FGF activity (Erm, PEA3) and several modulators of FGF activity (Sprouty2, Sprouty4, SEF). Similar to their *drosophila* counterpart, zebrafish sprouty2 and sprouty4 act as feedback-induced inhibitors of FGF signalling. Most importantly, inhibition of sprouty2 and sprouty4 by anti-sense morpholinos causes a dorsalization of the embryo, providing evidence that these factors are required *in vivo* to modulate dorsalizing FGF signals.

We have further shown that an additional member of the FGF8 synexpression group, the novel transmembrane protein SEF (for Similar Expression as Fgf), encodes a novel feedback-inhibitor of FGF signalling. To study the mode of action of SEF, we have analysed the signalling machinery that transduces the effect of FGF8, and found that its dorsalizing

activity is mediated through the activation of the RAS/MAPK-pathway. Epistasis analysis shows that SEF interferes with signal transduction between MEK and MAPK. As SEF is a transmembrane protein, it is likely to exert this action indirectly, maybe through the activation of MAPK-inhibitors. In accordance with such a model, first experiments show that overexpression of a MAPK-phosphatase causes developmental alterations that are similar to those induced by SEF.

In conclusion, we have shown that during the dorso-ventral patterning of the early zebrafish embryo, FGF signalling is required to restrict the expression of ventralizing BMPs. Correct dosage of dorsalizing FGF activities is achieved through the action of sprouty2 and sprouty4 as well as the novel feedback-inhibitor of RAS/MAPK-signalling SEF.

Robo receptor trafficking and midline crossing in *Drosophila*

Krystyna Keleman, Giorgio Gilestro and Barry J. Dickson

Axons in the *Drosophila* CNS receive conflicting signals from the midline: Netrin attracts them by signalling through the receptor Frazzled/DCC, while Slit repels them by activating Robo receptors. For commissural axons, Comm resolves this conflict by downregulating Slit repulsion. It selectively sorts newly-synthesized Robo, and not Frazzled, for lysosomal degradation.

Comm is active only in commissural neurons, and only as they grow across the midline. So during this brief window, Netrin attraction wins out over Slit repulsion. In ipsilateral neurons and post-crossing commissural neurons (or in all neurons in a comm mutant) Robo is instead sorted to the growth cone. Repulsion now wins over attraction, and these axons cannot cross the midline.

The function of Comm in midline crossing is therefore critically dependent on its ability to distinguish the two receptors, so that it selects Robo but not Frazzled for sorting from the trans-Golgi network to lysosomes.

Genetic and biochemical experiments revealing how Comm makes this distinction will be presented.

Membrane raft disruption at the basis of amyloid deposition in Alzheimer's disease patients with ApoE4 allele

Maria Dolores Ledesma, Cristian Galvan, Jose Abad Rodriguez, Andre Delacourte, and Carlos G. Dotti

The molecular mechanisms underlying the role of ApoE4 as a risk factor in Alzheimer's disease (AD) are not well established. In this work we show that the hippocampal membrane of ADApoE4 patients has low levels of cholesterol and paucity of raft microdomains. These patients also present a reduced membrane binding of plasminogen and low levels of the amyloid-degrading enzyme plasmin. Since identical deficits occur in hippocampal neurons in culture treated with membrane cholesterol-reducing drugs we propose that defects in membrane raft composition and organization are at the base of decreased degradation of amyloid peptide in ApoE4 carriers. The observed raft reduction could also result in alterations of other raft-mediated functions that could account for different symptoms of the disease.

Contribution of wingless and dishevelled to dorsal closure of the *Drosophila* embryo

Véronique Morel and Alfonso Martinez-Arias

During the first half of embryogenesis, the epidermis of the *Drosophila* embryo exhibits a hole on its dorsal side that is covered by the amnioserosa, an epithelium of large flat polyploid cells that will not contribute to the larva. As germband retracts, coordinated cell shape changes in the epidermis and the amnioserosa result in the progressive closure of the hole. By the end of this process, known as dorsal closure (DC), the two dorsal most rows of cells, the leading edge (LE) cells, meet, leading to the generation of a continuous dorsal epidermis. This process provides a good model system to study the behaviour of epithelial sheets during morphogenetic processes and wound healing (1).

Failures in DC lead to a dorsal hole, and with this criterion a number of genes have been identified involved in the mechanics of the process. The study of DC has thus far focused on the function of JNK and, to a much lesser degree, the contribution of some cytoskeletal components and their regulators to this process (2). However, live imaging of DC (3-4) and a recent study of the molecular organisation of the LE cells, conducted in the laboratory, have revealed a more complex landscape and provided a new framework to think about DC (5). DC begins as the LE cells elongate in the dorso-ventral axis and their microtubules organise into apical bundles along the same axis. Simultaneously, actin accumulates at the side of contact with the amnioserosa, first in thick aggregates at the border between LE cells, which have been dubbed Actin Nucleating Centers (ANCs) (5), and later all along the LE which then develops dynamic actin protrusions (6). The appearance of ANCs and the onset of actin dynamics correlates with the progressive clearance from the LE of apical proteins associated with Adherens Junctions proteins like Bazooka/Par-3, acatenin and Canoe, as well as baso-laterally localised proteins e.g. Discs large and Fasciclin III (5). We also observed that mutants for wingless or dishevelled, which show defects in DC, are also characterised by an abnormal polarisation of the LE cells, suggesting that this polarisation is of importance for the dorsal closure to proceed normally.

The role of wingless and dishevelled in dorsal closure and in polarisation of the LE cells will be described here.

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Competition for extracellular growth factors at the endocytic pathway

Eduardo Moreno and Konrad Basler

We have recently proposed (Moreno et al. 2002) that during tissue growth, cells compete for the internalization of extracellular factors required for their growth and survival. Cells unable to obtain sufficient quantities of survival factors undergo apoptosis and are eliminated from the tissue. This competition for extracellular factors is the mechanism by which weaker cells are eliminated from a growing population in *Drosophila*, and might serve as a general method of controlling cell number and optimizing tissue fitness, and hence organ function, in other animals. I will present new work about the role of the endocytic pathway during competition for the internalization of extracellular survival factors in *Drosophila*.

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Functional analysis of Rab5 effectors in *Drosophila* wing development

Giovanna Mottola

The small GTPase Rab5 is a crucial regulator of endocytosis. It controls internalization of endocytic vesicles at the plasma membrane, fusion of these vesicles with early endosomes and homotypic fusion between early endosomes (1,2). It also modulates the motility of early endosomes along microtubules (3) and participates in EGF signalling pathway (4,5). We purified a large number of cytosolic Rab5 effectors, demonstrating that the multifunctional activity of Rab5 depends on its ability to recruit these proteins to the membrane where it localizes (6,7). The effectors characterized so far function in a cooperative fashion and are restricted to a specific sub-domain of the early endosome. Beside studies in cultured cells, the function of Rab proteins and their effectors in an organism and during development has been poorly investigated. The aim of our project is to study the physiological role of two Rab5 effectors *in vivo*. We are focusing on Rabenosyn-5, which regulates two consecutive steps of endocytic trafficking (endocytosis and recycling), and a novel, yet uncharacterised complex of 5 proteins. Preliminary data in mammalian cells suggest that they localise to distinct endosomes or sub-compartments of early endosomes. As a model system we are using *Drosophila*, since recently a great deal of exciting results have been obtained on the role of endocytosis in many developmental processes, such as generation of morphogen gradient in wing imaginal discs, synaptogenesis and signalling (8,9,10). In order to study the intracellular localization and function of Rabenosyn-5 and the new complex in endocytic trafficking and wing development, we are generating mutant flies overexpressing or lacking each of these effectors.

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Control of Dpp gradient formation in the *Drosophila* wing

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In *Drosophila*, the TGF-β homolog Decapentaplegic (Dpp) functions as a morphogen required to specify cell fates along the anterior/posterior (A/P) axis of the wing. Dpp is expressed in a stripe of anterior wing cells at the A/P compartment boundary. It organizes wing patterning by inducing in a concentration-dependent manner target genes such as *spalt* (*sal*) and *optomotorblind* (*omb*). Recently, our lab showed that movement and gradient formation of Dpp is mediated by planar transcytosis initiated by dynamin-dependent endocytosis. The range of Dpp activity is limited by intracellular endocytic trafficking controlled by Rab proteins. We propose a model where the gradient is formed via intracellular trafficking of the ligand in receiving cells with the gradient slope controlled by endocytic sorting of the morphogen toward recycling versus degradation (Entchev et al., 2000). To determine which endosomal compartments are involved in morphogen traffic in imaginal disc cells, we have generated affinity purified antibodies which specifically recognize Rab5, Rab11 and Rab7, small GTPases which control the recycling and degradation of endocytic cargo, respectively. We determined GFP-Dpp localization in this endosomal compartments defined by Rab localization at the receiving cells. In order to test the planar transcytosis model, which assumes that internalized Dpp is resecreted from the receiving cells, we are currently analyzing whether internalized Dpp is again exocytosed by uncaging a Dpp chimera upon passage through the endosome at the receiving tissue.

Function of lipid binding proteins in clathrin mediated endocytosis

Peter, BJ, Mills, IG, Praefcke, GJK, Vallis, Y, Evans, PR, and HT McMahon

Clathrin mediated endocytosis is an essential process by which cells take up nutrients and signals from their environment. In addition to clathrin, it requires the participation of adapter protein complexes (APs), Phosphotidyl inositol (4,5) bisphosphate (PIP2), and a host of other proteins. Using immunofluorescence, electron microscopy and *in vitro* binding assays, we have determined that the protein epsin plays a role in bending the membrane during the early stages of endocytosis (1). However, mechanical processes such as changes in membrane shape, clathrin polymerisation and vesicle scission must be temporally and spatially coordinated in order to effect vesicle budding. The mechanism of regulation of epsin activity and that of other endocytic proteins remains unclear. Dynamin 1, amphiphysin 1 and 2, epsin, eps15, synaptojanin, and AP180 are all dephosphorylated during endocytosis of synaptic vesicles (2). Currently we are trying to determine the effects of epsin phosphorylation. In addition, we are studying the function of epsinR (also known as enthoprotin (3) and Clint (4)), which we find plays a role in vesicle trafficking from the trans-golgi network (TGN). By studying the function of epsin and similar lipid binding proteins, we hope to determine the mechanism of vesicle formation in endocytosis.

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Functional interaction of *Drosophila* aPKC with Crumbs in the control of epithelial cell polarity

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The establishment of apical-basal polarity in vertebrate and invertebrate epithelial cells requires the evolutionarily conserved Par-3/Par-6/aPKC complex. In the *Drosophila* embryo, function of the Crumbs/Discs lost/Stardust complex is also necessary. Since absence of either complex similarly disrupts apical-basal polarity, we have examined whether there is a functional relationship between them. Here we show the colocalization of both complexes and that they depend on each other for their function and subcellular localization. We also provide evidence for the requirement of the kinase activity of Drosophila aPKC (DaPKC) for the establishment of epithelial polarity. Moreover, in an *in vitro* system, DaPKC, Dlt and the intracellular domain of Crb physically interact and this promotes aPKC-dependent Crb phosphorylation. Thus, our results point to Crumbs as the first identified phosphorylation target of DaPKC.

Ptc mediated internalisation of Hh is not required for Hh signalling

Carlos Torroja

The Hedgehog (Hh) morphogenetic gradient controls multiple developmental patterning events in *Drosophila* and vertebrates. The receptor of Hh, Patched (Ptc), controls both Hh signalling and Hh diffusion. This paper reports how endocytosis regulates Hh concentration and activities in the wing imaginal disc. Ptc limits the Hh gradient by internalising Hh through endosomes in a dynamine-dependent manner, and then targeting it for lysosomal degradation. We describe here a Ptc mutant (ptc^{IIR87}) that cannot block Hh diffusion because it does not sequester and internalise Hh. Nevertheless, this mutant responds perfectly to Hh, indicating that Hh internalisation mediated by Ptc is not required for Hh signalling. In this mutant, and in the absence of Ptc, Hh was still found in internal vesicles in Hh-receiving cells, indicating that another internalisation process must exist.

Regulation of cell death and survival during inner ear development

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Nerve growth factor induces cell death in organotypic cultures of otic vesicle explants with a restricted pattern that reproduces the in vivo pattern of apoptosis occurring during inner ear development. Binding of nerve growth factor to its low affinity p75 neurotrophin receptor is essential to achieve the apoptotic response. Blockage of binding to p75 receptor neutralized nerve growth factor-induced cell death, as measured by immunoassays detecting the presence of cytosolic oligonucleosomes and by TUNEL assay to visualize DNA fragmentation. Nerve growth factor also induced a number of cell death-related intracellular events including ceramide generation, caspase activation and poly-(ADP ribose) polymerase cleavage. Again, p75 receptor blockade completely abolished all of them. Concerning the intracellular pathway, C_2-ceramide increase depended on initiator caspases while its actions depended on both initiator and effector caspases as shown using site-specific caspase inhibitors. Conversely, insulin-like growth factor-I, which promotes cell growth and survival in the inner ear, abolished apoptosis induced by nerve growth factor. Insulin-like growth factor cytoprotective actions were accomplished, at least in part, by decreasing endogenous ceramide levels and activating Akt. Taken together, these results strongly suggest that regulation of nerve growth factor-induced apoptosis in the otocysts occurs via p75 receptor binding and it is strictly controlled by the interaction with survival signalling pathways.

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