Instituto Juan March
de Estudios e Investigaciones

140 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

# Regulation and Functional Insights in Cellular Polarity

Organized by

A. R. Horwitz and F. Sánchez-Madrid

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A. Bretscher
P. Chavrier
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# Introduction

A. R. Horwitz and F. Sánchez-Madrid

Cell polarization and the establishment of functionally specialized domains plays a pivotal role in many cellular proceses such as vectorial transport of molecules, cell division and differentiation, migration and directional movement of the cells in a chemotactic gradient and activation of the immune response. Polarization may be constitutive, such as in neurons or epithelium, or inducible, such as in mating or budding yeast or during cell migration. This is a complex phenomenon in which the interplay among cell cytoskeletal components, extra- and intracellular signals and organelle and membrane reorganization is crucial to achieve a correct cell shape change.

The aim of this meeting has been to compare recent advances in different fields related to cell polarization. Research in yeast provide a basic tool in which to dissect genetics of molecules involved in the generation of specific compartments within the cell and other cycle-related phenomena, such as budding or pheromone mating as well as fission. On the other hand, some of the discussion has involved the polarized secretion of molecules and its impact in the development and maintenance of cell polarity, with new data implicating the role of the actin cytoskeleton and the machinery involved in vesicle formation and trafficking. The role of membrane composition and dynamics has also been described, with special attention to the role of lipid rafts in the generation of cell compartments and the spatial regulatory role of rafts in activation of signaling components depending on the presence of different molecules in this lipid domains. Cell migration is an active field of study in which the development of polarity is a requisite for cell movement. Recent advances in genes controlling cell adhesion and migration in different cell models, such as *Dictyostelium*, fibroblasts, neutrophils or lymphocytes have been reviewed. Finally, the establishment of cognate immune cell-cell interactions and the regulatory molecules has been discussed.

A. R. Horwitz and F. Sánchez-Madrid

# Session 1: Establishment and maintenance of cell polarity
## Chair: Kai Simons

## Roles of budding yeast Cbk1p kinase in cell morphogenesis

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Accurate spatial and temporal control of processes that determine cell morphology is important for productive cell growth and division. Budding yeast cells lacking the Ndr/LATS-related protein kinase Cbk1p fail to sustain polarized growth during early bud morphogenesis and mating projection formation. Cbk1p is also required for Ace2p-dependent transcription of genes involved in mother/daughter separation following cytokinesis. We showed that the conserved protein Mob2p is required for Cbk1p function. Mob2p and Cbk1p physically associate and interdependently localize to incipient bud sites and the daughter cell cortex during bud emergence and growth. Mob2p is required for both full Cbk1p kinase activity and Cbk1p hyperphosphorylation in vivo. At the end of mitosis, Mob2p and Cbk1p localize to the bud neck and daughter cell nucleus; we found that Ace2p similarly localizes to daughter cell nuclei. Restriction of Ace2p to daughter cell nuclei requires Mob2p, Cbk1p, and a functional nuclear export pathway. Nuclear localization of Mob2p and Ace2p do not occur in mob1-77 mutants, which are defective in Mitotic Exit Network (MEN) signaling, even when mob1-77 cell cycle arrest is bypassed. Collectively, these data indicate that Mob2p-Cbk1p has three functions: 1) to maintain polarized cell growth; 2) to prevent the nuclear export of Ace2p from the daughter cell nucleus following mitotic exit and 3) to coordinate Ace2p-dependent cell separation with MEN activation.

# How yeast assembles and utilizes a polarized actin cytoskeleton for cell growth and organelle segregation

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In yeast the actin cytoskeleton is known to play a critical role in directing polarized growth and the polarized segregation of organelles during the cell cycle. Yeast contains at least three actin-based structures: cortical patches, actin cables and the contractile ring. Previously it has been shown that actin cables play a key role in many, if not all, aspects of cell polarity (1). We have shown by *in vivo* visualization that polarized delivery of post-Golgi secretory vesicles along actin cables is mediated by the myosin-V encoded by the *MYO2* gene (2, 3). This same myosin is responsible for vacuole (4, 5) and late Golgi element segregation (6), as well as initial orientation of the nucleus in preparation for mitosis (7). We have now investigated the mechanism of assembly of actin cables and found that it depends on yeast formin function. Yeast contains two formins, Bni1p and Bnr1p, either of which can provide an essential function. We have generated and characterized conditional *bni1* alleles in a *bnr1*Δ strain. Loss of Bni1p function is accompanied by rapid loss of actin cables, which leads to loss of polarized growth as well as misorientation of the mitotic spindle. Restoration of Bni1p activity was able to restore actin cables in an Arp2/3-independent manner. Analysis of fragments derived from the C-terminal half of Bni1p indicate that this region has the ability to direct the assembly of actin filaments *in vivo*, and that this activity is normally negatively regulated by the N-terminal domain (8, 9). Analysis of fragments of Bni1p show that the FH2 domain can induce the assembly of actin structures *in vivo*, and drive the assembly of actin filaments *in vitro*. Moreover, the FH2 domain binds to the barbed end of assembling filaments, suggesting that it functions *in vivo* to assemble polarized actin filaments that can then serve as substrates for the vectorial delivery of components by myosin-V.

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## Mechanisms of polarized membrane traffic in epithelial cells

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The ability of membrane proteins to reach the apical or basolateral domains of polarized epithelial cells often depends on the presence of distinctive sorting signals. Signals for basolateral transport are the best characterized both structurally and mechanistically. They reside in the cytoplasmic domains of many types of membrane proteins and involve sequence motifs that are at least superficially related to motifs responsible for endocytosis via clathrin-coated pits. Indeed, a major class of these signals interacts selectively with an epithelial cell-specific clathrin adapter complex termed AP-1B. This complex is distinguished from the ubiquitously distributed AP-1A complex in structure (it contains the epithelial cell specific 50 kD μ subunit μ1B), in specificity (AP-1A and AP-1B cargo are entirely distinct), and in function (AP-1B cannot substitute for AP-1B in mediating proper transport between the TGN and endosomes). AP-1A and AP-1B also comprise largely non-overlapping populations of clathrin-coated vesicles.

Genetically, we have been able to show that recognition of AP-1B-dependent signals is absolutely required for targeting of membrane proteins to the basolateral surface. In addition, it now also appears that regulation of this interaction can explain why some basolateral proteins, such as the adhesion molecule L1/Ng-CAM, engage in efficient transcytosis to the apical surface. Interestingly, however, not all basolateral proteins depend on AP-1B for basolateral transport. Proteins bearing dileucine-containing targeting motifs, for example, reach the basolateral surface even in the absence of μ1B expression.

The identification of AP-1B as an essential component in basolateral transport has allowed us to begin locating and characterizing other components that are required for this pathway. Thus far, we have found that expression of mutant alleles of Cdc42 or Rab8 selectively inactivates the transport of AP-1B-dependent cargo, both transport to the apical surface and even basolateral transport of AP-1B-independent (dileucine signal-containing) cargo continued unabated. Interestingly, Cdc42 and Rab8 expression seemed to cause a selective disorganization of the TGN, altering those regions containing AP-1B-dependent cargo but not AP-1A-dependent cargo, or the cisternal Golgi.

The AP-1B pathway was shown to be mechanistically distinctive in other respects as well. Expression of μ1B was found to selectively recruit at least some exocyst components to the TGN region of epithelial cells including Sec6, Sec8, and Exo70. The sites to which these elements were recruited moreover appeared physically distinct from areas of the TGN-region containing AP-1A or related cargo proteins such as TGN38 or furin. These results suggest that there is a spatial segregation of sorting events in the TGN which allows for the accurate assembly of basolateral vesicles. This mechanism may help ensure that basolateral transporters contain both the proper cargo and the cytosolic components required for proper targeting to and fusion with the basolateral plasma membrane. Conceivably, segregation is triggered by the initial binding of AP-1B to the TGN.

# Regulation of protein transport from the Golgi complex to the endoplasmic reticulum by Cdc42 and N-WASP

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Actin is involved in the organization of the Golgi complex and Golgi-to-ER protein transport in mammalian cells. Little, however, is known about the regulation of the Golgi-associated actin cytoskeleton. We provide evidence that Cdc42, a small GTPase that regulates actin dynamics, controls Golgi-to-ER protein transport. We located GFP-Cdc42 in the lateral portions of Golgi cisternae and in COPI-coated and non-coated Golgi-associated transport intermediates. Overexpression of Cdc42 and its activated form Cdc42V12 inhibited the retrograde transport of Shiga toxin from the Golgi complex to the ER, the redistribution of the KDEL receptor, and the ER accumulation of Golgi-resident proteins induced by the active GTP-bound mutant of Sar1 (Sar1[H79G]). Co-expression of wild type or activated Cdc42 and N-WASP also inhibited Golgi-to-ER transport but this was not the case in cells expressing Cdc42V12 and N-WASP(?WA), a mutant form of N-WASP that lacks Arp2/3 binding. Furthermore, Cdc42V12 recruited GFP-N-WASP to the Golgi complex. We therefore conclude that Cdc42 regulates Golgi-to-ER protein transport in an N-WASP-dependent manner.

## Microtubule architecture and spatial regulation in the fission yeast cell

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A fundamental question in cell biology asks how positional information is generated within the cell. How are specific sub-cellular domains established and maintained, and how are cellular organelles and structures positioned?

By developing and applying advanced fluorescence imaging and analysis techniques to living yeast cells expressing GFP fusion proteins, we established that the interphase nucleus is dynamically positioned at the center of the cell by a balance of pushing forces produced by cytoplasmic microtubule (MT) bundles emanating from multiple novel MT-organizing-centers, the iMTOCs, attached to the nuclear envelope. We also characterized rsp1, whose gene product is an iMTOC component required for proper iMTOC organization and function. rsp1 mutant cells have striking defects in interphase MT organization and nuclear and septum positioning. Instead of multiple iMTOCs and multiple MT bundles, rsp1 cells maintain a single, large MT "aster" with only one iMTOC, the spindle pole body. rsp1? cells have fewer MT bundles than do wildtype cells, whereas over-expression of rsp1+ results in cells with a single large MT bundle.

Our studies established the architectural and dynamic parameters of the MT cytoskeleton in fission yeast. Our findings lead to a model in which the MTs, arranged in novel bilaterally-symmetric, dynamic and rigid bundles with their plus ends facing the cell tips and their overlapping minus ends attached to the nuclear envelope at the iMTOCs, help define discrete spatial domains within the cell. In this model, nuclear position dynamically marks the cell middle, while dynamic interactions between MT plus ends and the distal cell cortex mark the cell tips, the sites of polarized cell growth.

## Session 2: Membrane organization and scaffolding in cell polarization
## Chair: David G. Drubin

## Lipid rafts 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 investigated the polarization process during yeast mating. This process involves a reorganization of the membrane which results in the clustering of rafts at the tip of the mating projection. We have demonstrated a role for lipid rafts in polarized localization of proteins required for mating.

## The MAL protein family as machinery for polarized transport

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Apical targeting of newly synthesized protein places in polarized epithelial cells by direct transport from the Golgi and/or by an indirect pathway known as transcytosis, which involves the delivery of the protein to the basolateral membrane and its subsequent transport to the apical surface. Recruitment of specific proteins into specialized glycolipid and cholesterol-enriched membrane microdomains or rafts was proposed to explain the segregation and subsequent transport of newly synthesized apical proteins by the direct route (1). Although the lipid composition of the rafts provides the basis for the specificity of protein recruitment, it was postulated that rafts require specialized protein machinery to function as a transport route. The following requirements are predicted for the integral membrane elements of this machinery: 1) expression in polarized cell types, 2) presence in lipid rafts, 3) post-Golgi distribution, and 4) participation in the sorting process.

MAL is an integral membrane protein of 17 kDa that is expressed in polarized epithelial cells in many tissues (e.g., kidney, thyroid, intestine, stomach), and also in myelin-forming cells and T lymphocytes (requirement 1). MAL expression has been found in representative cell lines of some of these tissues, including the epithelial renal MDCK and thyroid FRT cell lines and Jurkat T cells. MAL selectively resides in lipid rafts in all these cell lines (requirement 2). Endogenous MAL is localized at steady state predominantly in a compartment located to the apical zone of polarized epithelial cells (requirement 3). The elimination of the endogenous MAL protein in MDCK cells blocks transport of integral membrane proteins, GPI-anchored proteins and secretory proteins to the apical surface (requirement 4) (2-4). The fulfillment of the four requirements demonstrates an essential role for MAL as an element of the machinery for apical transport in MDCK cells.

MAL is the founder member of a family of proteins, the MAL protein family, with structural and biochemical similarities (5). As we considered plausible the hypothesis that, similar to MAL, members of the MAL family play a role acting as machinery for other raft-mediated membrane trafficking pathways, we have undertaken the biochemical and functional characterization of novel members of the MAL family. BENE, the second member of the MAL family to have been characterized to date, also resides exclusively in raft membranes (6). Our studies point out for a role of the MAL family of proteins as machinery for specialized membrane trafficking pathways.

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## Lessons from the dialogue between cell polarization and motility

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During cell migration, cells become polarized, meaning that they acquire and maintain spatial as well as functional asymmetry. At the front of the cell, the plasma membrane must extend forward and adhere to the substrate, whereas the rear of the cell must contract and detach from the substrate in a coordinated manner. This segregation of functions is possible since signaling pathway components that control actin polymerization and cell polarization are asymmetrically targeted to and activated in the plasma membrane after chemoattractant stimulation. These signaling events are initiated locally in the region of the chemotactic cell that faces the higher concentration of attractant (leading edge); negative signals may be elicited simultaneously to prevent their activation in inappropriate parts of the cell. Although the asymmetrical targeting of signaling molecules observed in migrating cells may account for the establishment and maintenance of cell polarity, the mechanisms controlling the spatial localization of these molecules during polarization and locomotion have not been described. Here, new evidence will be discussed on how moving cells regulate the activation of specific signaling pathways in time and in space. On the one hand, the regulation of monomeric vs. dimeric states of chemoattractant receptors may be a fundamental mechanism by which the cell controls the activation of specific signaling pathways during the migration process. On the other, evidence will be presented indicating that cholesterol- and glycolipid-enriched raft membrane domains may serve as platforms on which these interactions are regulated. A model will be presented in which the asymmetric distribution of raft domains in moving cells may be a basic mechanism to spatially regulate the activation of specific signaling pathways involved in cell polarization and locomotion.

## Mechanism of sorting of GPI-anchored proteins in polarized epithelial cells

Simona Paladino and Chiara Zurzolo

The raft model postulates that the selective apical delivery of GPI-proteins in epithelial cells depends on their incorporation in detergent-insoluble microdomains (DRM or rafts). To study the mechanism of sorting and trasport of GPI-proteins we stably transfected a chimeric GFP-GPI protein in FRT and MDCK cells. GFP-GPI is present on the basolateral domain in FRT cells and prevalently on the apical surface in MDCK cells.We analyzed the transport to surface in living cells using a digital system of image analysis after block at 20°C in the Golgi and release at 37°C. Upon exit from the TGN, GFP-GPI protein displayed similar kinetics of Golgi emptying and surface arrival in both cell lines. To determine the role of rafts in the delivery to surface we perturbed rafts formation by cholesterol depletion. In these conditions the transport to the surface slows down in FRT and it is completely blocked in MDCK cells. Interestingly we found that after block at 20°C the amount of GFP-GPI associated with DRM is greatly increased both in FRT and in MDCK cells. These data indicate that rafts are likely to be present in the TGN but do not represent a selective mechanism for apical sorting of GPI-proteins.

We hypothesized that apical sorting is mediated by stabilzation of proteins into rafts and/or by rafts coalescence. Preliminary data indicate that this could be achieved by oligomerization of apically sorted GPI-proteins. Indeed while in FRT cells GFP-GPI is present almost esclusively as monomer or dimer, in MDCK cells GFP-GPI forms high molecular weight complexes. We are currently analyzing the composition of these complexes and their possible involvment in apical sorting.

## Regulation of cell polarity by Gef1p, a new guanine nucleotide exchange factor specific for Cdc42p in fission yeast

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*Schizosaccharomyces pombe* cells are rod-shaped and undergo three main morphological transitions in a dynamic process coupled to cell cycle progression. After cytokinesis by medial fission, the newly divided cells grows by apical extension of the "old end" that existed prior to septation. This monopolar growth continues until early G2 phase. At this time, a transition to bipolar growth occurs by using the new end originated during cell division . Finally, when the cell reaches its maximal size, elongation ceases and mitosis occurs followed by the formation of the septum and cell separation.

Cdc42 GTPase plays a critical role in the establishment of polarity in most eukaryotic cells. In *S. pombe*, cdc42+ is an essential gene required for growth and maintenance of cell morphology. The only described guanine nucleotide exchange factor (GEF) for Cdc42p is Scd1p/Ral1p, an homologue to *S. cerevisiae* Cdc24p. scd1 deletion causes rounded cells but is not lethal suggesting that Scd1p is not the sole physiological GEF for Cdc42p.

We have identified a new specific Cdc42-GEF, named Gef1p. Inactive Cdc42p binds to the C-terminal region of Gef1p where the consensus DH domain is located. Overexpression of gef1+ increases the active form of Cdc42p, and causes changes in cell morphology and cell size similar to those caused by overexpression of the constitutively active Cdc42G12V allele. gef1+ deletion is viable in wild type cells but the double mutant gef1D scd1D is lethal, suggesting that they share an essential function as GEFs for Cdc42p. However, they do not have overlapping functions in the control of cell polarity. Gef1p localizes to the septum and gef1D causes defects in septum formation. Genetic evidence indicates that Gef1p interacts with Shk1p/Pak1p, one of the two Cdc42p effector kinases which is involved in the switch from monopolar to bipolar growth.

## Session 3: Signaling and cell polarity
## Chair: J. Victor Small

# Rho family proteins in cell adhesion, cell migration and tumor formation

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Rho-like GTPases, including Cdc42, Rac and RhoA, control signalling pathways that regulate the actin cytoskeleton as well as gene transcription (1,2). In particular, Rho-like proteins act as key control molecules in signaling pathways that determine the reorganization of the actin cytoskeleton in response to receptor stimulation. Similar to Ras proteins, Rho-like proteins cycle between an active GTP-bound state and the inactive GDP-bound state. The dynamic cytoskeletal changes regulated by Rho-like GTPases determine the morphology, adhesion and motility of cells, processes required in embryonic development and in invasion and metastasis of tumor cells.

In previous studies, we have identified the invasion-inducing Tiam1 gene, which encodes an activator (GEF) of the Rho-like GTPase Rac (3,4). Similar to constitutively active V12Rac, Tiam1 induces an invasive phenotype in T-lymphoma cells. Also activated V12Cdc42 induces invasion of T-lymphoma cells which is not caused by Cdc42-mediated activation of Rac but presumably by the stimulation of common downstream pathways of Cdc42 and Rac. Activated V14RhoA potentiates invasion but fails by itself to mimic Rac and Cdc42. The Rho-like GTPases thus cooperate in the acquisition of an invasive phenotype of lymphoid tumor cells (5).

In epithelial carcinoma cells, invasion and metastasis is often associated with reduced E-cadherin-mediated cell-cell adhesion. Ectopic expression of Tiam1 in epithelial cells inhibits HGF-induced cell scattering and cell migration by increasing E-cadherin-mediated cell-cell adhesion. Increased Tiam1-Rac signaling also inhibits invasion and migration of fibroblastoid Ras-transformed MDCK cells by restoring E-cadherin-mediated adhesions and an epithelial phenotype (6). Interestingly, Tiam1/Rac-induced cellular responses with respect to cell-cell adhesion and cell migration are dependent on integrin-mediated cell substrate interactions and the cell type studied (6,7). Migration of epithelial cells is thus determined by a balance between invasion-inhibitory cell-cell interactions and invasion-promoting cell-substrate interactions, both mediated by PI3-kinase-regulated Tiam1-Rac signaling.

The proto-oncogene Ras is frequently mutated in epithelial tumors, resulting in uncontrolled growth and transition towards an invasive, mesenchymal phenotype due to loss of E-cadherin-mediated adhesions. We found that sustained oncogenic Ras signaling in epithelial cells permanently downregulates Rac and upregulates Rho activity, which is accompanied by epithelial-mesenchymal transition (8). Oncogenic Ras provokes changes in Rac and Rho activity through sustained activation of the Raf/MAP-kinase pathway, which causes transcriptional downregulation of Tiam1, an activator of Rac. Reconstitution of Rac activity by exogenous expression of Tiam1 decreases Rho activity and restores the epithelial phenotype in mesenchymal V12Ras or RafCAAX-transformed cells. Our findings identify the inactivation of Rac by transcriptional downregulation of an exchange factor as an important mechanism for Ras-induced transformation of epithelial cells.

All these in vitro data suggest that Rac may play a role in the formation and progression of epithelial tumors in vivo. To study this directly, we have generated mice lacking the Rac-specific activator Tiam1 using gene targeting. Genotyping revealed the successful generation of heterozygous (Tiam1$^{+/-}$) and homozygous Tiam1$^{-/-}$ mice and Western blot analysis confirmed the absence of Tiam1 protein in different organs of Tiam1$^{-/-}$ mice. Consistent with the role of Tiam1, reduced Rac activity was detected in Tiam1-deficient embryonic stem cells and in primary keratinocytes derived from Tiam1$^{-/-}$ mice. Although Tiam1 is expressed during embryonic development, Tiam1$^{-/-}$ mice surprisingly develop, grow and reproduce normally. Compensation by other GEFs capable of activating Rac, such as Sos, Vav, Pix, Trio, SWAP-70, and Tiam2, may play a role in suppressing a developmental phenotype.

In mouse skin, Tiam1 is present in basal and suprabasal keratinocytes of the interfollicular epidermis and in hair follicles where it is predominantly expressed in the infundibular portion. Tiam1 was not detected in skin derived from Tiam1$^{-/-}$ mice, consistent with a reduced basal Rac activity found in primary keratinocytes isolated from Tiam1$^{-/-}$ mice. Therefore, skin tumors were initiated in wild-type (Tiam1$^{+/+}$) and Tiam1$^{-/-}$ littermates by application of a two-stage chemical carcinogenesis protocol (9,10). This protocol entails tumor initiation in epidermal keratinocytes by a single treatment with the carcinogen DMBA, which invariably induces oncogenic activation of the c-Ha-Ras gene. Subsequent repeated treatments with the tumor promoter TPA result in the outgrowth and progression of initiated cells. Results on the role of Tiam1-mediated Rac signaling in the initiation, the promotion and the progression of skin Ras-induced skin tumors will be discussed.

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# Dissection of pathways mediated by Rho effectors; implications in cell polarization and migration

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Cells undergoing directional migration exhibit a polarized morphology with a broad lamella at the leading edge and a trailing tail at the rear. While this morphology depends on spatiotemporal organization of different types of the actin cytoskeleton, microtubules (MTs) also work critically in establishing this polarity, suggesting that MTs and actin cytoskeleton are coordinately regulated in migrating cells. While accumulating evidence suggests that the Rho family small GTPases participate in these shape changes of migrating cells, how these GTPases regulate spatiotemporally change of the cytoskeletons in migrating cells remains largely unknown. Involvement of Rho GTPases has been studied in one way in the in vitro wound-healing assay, which revealed the bimodal Rho actions in cell migration (1). Namely, when two doses of C3 exoenzyme, a Rho inactivator, was microinjected into migrating REF cells in this assay, the low dose that was sufficient to abolish stress fibers and focal adhesions did not affect cell migration, while the high dose completely inhibited cell migration. Furthermore, Y-27632, an inhibitor of a Rho effector, ROCK, accelerated the migration of the cells. We wondered that such bimodal Rho action may be derived from different actions the two Rho effectors mDia1 and ROCK exert in the cell. We previously found that ROCK induces cell contraction through enhancement of actomyosin contractility, while expression of a dominant active mDia1 aligns MTs in parallel to induce bipolar cell elongation and to orient actin bundles (2,3). In order to examine the interaction between these ROCK and mDia actions, we compared the effects of C3 exoenzyme with those of Y-27632 on responses of serum-starved Swiss 3T3 fibroblasts upon LPA stimulation (4). We have found that Y-27632 treatment specifically induced membrane ruffles and focal complexes in these cells. This Y-27632-induced membrane ruffle formation was suppressed by expression of N17Rac. Consistently, the amount of GTP-Rac increased significantly by Y-27632 in LPA-stimulated cells. Biochemically, Y-27632 suppressed tyrosine-phosphorylation of paxillin and FAK and not that of Cas. Inhibition of Cas phosphorylation with a Src inhibitor, PP1, or expression of a dominant negative Cas mutant inhibited Y-27632-induced membrane ruffle formation. Moreover, Crk-II mutants lacking in binding to either phosphorylated Cas or DOCK180 suppressed the Y-27632-induced membrane ruffle formation. Finally, expression of a dominant negative mDia1 mutant or disruption of MTs by nocodazole inhibited also the membrane ruffle formation by Y-27632. These results have thus revealed the dichotomy of Rho signaling. One is mediated by mDia, leading potentially to Rho-dependent Rac activation through Cas phosphorylation. The other is mediated by ROCK, which, when active, works to suppress the above mDia-mediated Rac activation. This dichotomy of Rho signaling may have several implications in cell migration and polarization. It may correspond to the bimodal function of Rho in cell migration. Facilitation of cell migration by Y-27632 may thus be due to activation of Rac through the mDia pathway. This dichotomy may also be involved in recycling of Rho GTPase cascade. Previously, the hierarchical activation of Cdc42 to Rac and then Rac to Rho was shown, but not a pathway from Rho to Rac or Cdc42 activation. It may also provide one mechanism for MT-dependent polarized growth. We have already noted that bipolar cell elongation of HeLa cells by active mDia1 is inhibited by

expression of dominant negative Rac mutant. This mechanism may also be involved in neurite outgowth in neuronal cells, that is now under analysis in our laboratory.

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# PI 3-kinases and Rho GTPases in cell adhesion and migration

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We are studying how signal transduction through PI 3-kinases and Rho family GTPases contributes to motile responses involving leukocytes, epithelial cells and endothelial cells. In response to tyrosine kinase receptor activition, we and others have previously shown that class 1a PI 3-kinases act upstream of Rac to induce lamellipodium extension and subsequent migration (1-4). Rho is also required for cell migration, and this reflects at least in part its effects on cell contractility and cell detachment (3). In contrast, the related protein Cdc42 is not required for migration of macrophages but for determining the direction of migration (chemotaxis) (3). Cdc42 induces extension of filopodia, and WASp is a downstream target of Cdc42 involved in stimulating actin polymerization via the Arp2/3 complex. We have found that WASp is tyrosine phosphorylated by the leukocyte-specific tyrosine kinase Hck, and have mapped the site of tyrosine phosphorylation. A WASp mutant where this tyrosine is mutated to glutamate (to mimic phosphorylation) can induce filopodium extension independently of Cdc42. This indicates that WASp is subject to multiple levels of regulation and that the involvement of Cdc42 depends on the stimulus.

In endothelial cells, cytokines such as TNFα induce a programme of transcriptional changes in, leading to enhanced transmigration of leukocytes in inflammatory responses. Transmigration is dependent initially on the binding of leukocytes to the endothelial cells, and this involves a number of receptors expressed on the endothelium, including ICAM-1, VCAM-1 and E-selectin. Inhibition of Rho proteins reduces monocyte adhesion and transmigration (6). We have found that adhesion of monocytes to endothelial cells or cross-linking of ICAM-1 activates RhoA in endothelial cells to induce stress fibre assembly, and also rapidly induces transcriptional changes. In contrast, the related ICAM-2 protein does not activate RhoA. The role of signalling between ICAMs and RhoA in leukocyte transmigration will be discussed.

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## Finding their way - role of PI3K in directional sensing

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Phosphatidyl inositol-3 kinase (PI3K) is a key regulator of chemotaxis in leukocytes and *Dictyostelium* cells. We have demonstrated that the downstream PI3K effectors Akt/PKB and PhdA are required for proper cell polarity by regulating 1) PAKa and myosin assembly and 2) actin polymerization, respectively, during chemotaxis. PKB activity is stimulated by chemoattractant signaling via a PI3K-dependent pathway and activates, through direct phosphorylation, PAKa, a PAK1 homologue, which, in turn, regulates myosin assembly, cortical tension, and retraction of the posterior rear body, all of which are essential for proper cell movement. PhdA, in turn, controls the spatio-temporal control of actin assembly. PKB and PhdA transiently and rapidly bind to PI3K products on the plasma membrane via their PH domains, which is dependent upon chemoattractant-mediated PI3K activation. These findings suggest that translocation of PH-domain-containing proteins to the leading edge may occur by localized activation of PI3K at this site on the membrane.

Using PI3K-GFP and PI3K-GFP deletion mutants, we have demonstrated that PI3K-GFP translocates to the membrane upon chemoattractant stimulation and localizes to the leading edge during chemotaxis. Expression of PI3K-CFP and PhdA-YFP enabled us to demonstrate simultaneous co-translocation of both proteins to the membrane. However, the kinetics of release from the plasma membrane do not coincide, suggesting that the adaptation pathways controlling these two processes may be distinct. Deletion analysis revealed that only the N-terminal region of PI3K, lacking the Ras binding domain, C2, and kinase domains, is required for the translocation. Translocation of N-terminal region, but not PhdA-YFP, was observed in *pi3k* null cells or wild-type cells treated with LY294001, a PI3K inhibitor, suggesting translocation of PI3K does not require PI3K activity. Furthermore, we show that the phosphoinositide 3' phosphatase PTEN attenuates downstream PI3K signaling (e.g. PKB/Akt activation). We show that PTEN is on the membrane of unstimulated cells and is transiently released from the membrane coincident with PH domain membrane localization. PTEN is reduced in concentration at the leading edge of migrating cells but not at other regions of the membrane. PTEN's subcellular localization during chemotaxis and redistribution in response to chemoattractants are consistent with a role in establishing and maintaining a phosphoinositide-enriched domain at the leading edge. PTEN is thus a component of the pathway that helps restrict PI3K-dependent pathways to the leading edge of cells.

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## Rho signalling via ROK to myosin-II mediates the recruitment of Arp2/3 complex and actin filaments during CR- phagocytosis, but not FcγR-phagocytosis

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Phagocytosis through Fcγ receptor (FcγR)- or complement receptor-3 (CR3) requires Arp2/3 complex mediated-actin filaments polymerisation, however the signalling mechanisms of each receptor are quite distinct. During FcγR-mediated phagocytosis Rac and Cdc42 are required for actin and Arp2/3 complex recruitment contrasting with the requirement of Rho for these proteins to be recruited to CR3-phagosomes. Since membrane protrusions do not occurr around the CR3-phagosome, unlike Fc-phagosome, our goal was to analyse whether the driving force for internalisation during CR3-uptake was provided by a contractile activity through the Rho > ROK > myosin-II pathway.

Inhibition of either ROK, MLCK or myosin-II caused a reduction of Arp2/3 complex and actin accumulation around bound particles leading to a reduction in phagocytic engulfment during CR3-phagocytosis. However inhibition of myosin-II during FcγR-mediated phagocytosis only reduced the uptake of particles, but did not affect the ability to recruit Arp2/3 c1mplex or actin.

We conclude that myosin-IIA activation by ROK is required for internalisation of particles by CR3, downstream of RhoA. Furthermore, ROK-myosin-II or MLCK-myosin-II activity is needed for the recruitment of Arp2/3 complex and F-actin during this process. On the contrary, FcγR-mediated phagocytosis requires MLCK-myosin-II for the uptake of particles, but independently of the association of Arp2/3 complex or F-actin to the phagosome.

## Lymphocyte polarization and motility: RhoA effectors involved in lymphocyte migration and cytosketal rearrangements

Miguel Vicente-Manzanares

Lymphocyte migration involves cell polarization in response to extracellular cues, such as chemoattractant gradients. Polarization requires cytoskeletal reorganization, in which Rho GTPases are likely candidates to coordinate such changes. We have addressed the role of RhoA and its downstream effectors p160ROCK and p140mDia1 in chemokine- dependent- and independent- cytoskeletal changes. The chemokine SDF-1 induces robust RhoA and ROCK activation as well as RhoA- and ROCK-dependent MLC phosphorylation in peripheral blood lymphocytes (PBL), although it seems not to play an essential role in SDF-1-induced short-term actin polymerisation. On the other hand, inhibition of RhoA and ROCK blocked SDF-1-induced lymphocyte migration and polarization and caused the appearance of abnormal, needle-like spikes, which were found to relate to tubulin stability. In addition, we have investigated the involvement of p140mDia1 in such processes in PBL. Strikingly, p140mDia1 was up-regulated during T cell activation and differentiation to T lymphoblasts. Confocal studies revealed that p140mDia1 localizes to the rear part of the leading edge of polarized lymphocytes, thus suggesting its involvement in actin-related processes behind the leading lamella. Finally, overexpression studies demonstrated an essential role for p140mDia1 in lymphocyte motility through regulation of the basal levels of F-actin. We propose a model of coordinated and spatial regulation of the lymphocyte cytoskeleton, migration and chemokine responses by RhoA effectors.

## Session 4: Polarization during cell migration
## Chair: John G. Collard

## New insights in "in situ" cell migration

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Cell migration is a complex, multi-step process that plays a central role a variety of normal and pathological processes including embryonic development, cancer, and tissue repair and regeneration. Functional and molecular polarity is an intrinsic feature of a migrating cell as it moves directionally toward its target. Adhesions form at the cell front and turnover at the cell rear while adhesive components are trafficking from the rear where they leave adhesions and toward the front where new adhesions form. By co-expressing pairs of adhesion molecules fused to GFP analogues, we have studied the mechanisms by which adhesions form and turnover and traffic into and out of adhesions. The data point to a sequential model for the assembly of adhesions and recently key regulators of adhesive turnover have been identified. GFP fusion proteins have also revealed two different modes for the trafficking of adhesive components. One is vesicle based and moves integrins from the leading edge and cell rear to the perinuclear region and from the perinuclear region to the base of the leading edge. We have also seen multi-molecular signaling complexes trafficking among the leading edge, adhesions, and a cytoplasmic compartment. The function of these GIT1, paxillin, and PAK containing complexes is location dependent; for example, they regulate the formation of protrusions at the leading edge and turnover when in adhesions.

These studies rely on dissociated cells growing on homogeneous, planar substrates in medium containing serum or purified growth factors. This geometry and environment differs from that of cells migrating in vivo. Therefore, we have developed methodology for visualizing GFP fusion proteins in migrating cells in vivo using 200-400μ slices from embryo or adult tissues from three systems: the migration of muscle precursors from somites to the limb, the migration of neuronal precursors along the rostral migratory stream, and the migration of granule cells in the cerebellum. These migrations are characterized by bursts of rapid migration and a single, highly directed protrusion. These protrusions are mediated by the local activation of Rac, and the polarity arises by signaling through chemoattractive receptors like DCC.

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# Microtubules determine the polarity of migrating cells via a cross-talk with substrate adhesions

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To invade new territory, a cell must protrude a front and, subsequently retract its rear. It must accordingly set up an asymmetric, or polarised form, whereby the protruding and retracting zones are more or less diametrically opposed. The procesess of protrusion and retraction are mediated by the actin cytoskeleton. But polarisation requires the cooperation of microtubules, since it is lost or impaired when microtubules are disassembled (Vasiliev, 1985). So how do microtubules influence cell polarity in migrating cells? Answers to this long standing question are coming from findings implicating microtubules in the spatial control of the adhesion patterns that cells develop with their substrate.

Initial indications that microtubules interface with the actin cytoskeleton at adhesion sites came from the observation in living fibroblasts showing that microtubules specifically target substrate adhesions (Kaverina et al 1998). It was further found that these targeting events could be followed by the turnover of adhesion sites, or their dislocation from the substrate (Kaverina et al, 1999). Given the dependence of adhesion site maintenance on contractility (Chrzanowska-Wodnicka and Burridge, 1996) it was speculated that microtubules destabilise adhesions by delivering signals that antagonise the contractility pathway. This contention was supported by the demonstration that dissociation of adhesion sites at the cell edge could be mimicked by the local application of inhibitors of actomyosin contractility (Kaverina et al., 2000).

The putative signal delivered to (or sequestered by microtubules) at adhesion sites has yet to be identified. However, results from experiments in which the activities of microtubule motors were inhibited, suggest that kinesin is involved in signal transmission. Thus, inhibition of kinesin-1 activity by injection into cells of a function blocking antibody produced the same changes as observed after microtubule disruption with nocodazole (Krylyshkina et al., 2001): namely, the depolarisation of cell shape as well as an increase in size of focal adhesions. The same effect was induced by the injection of a kinesin-1 heavy chain bearing a mutation in the motor domain that results in irreversible binding of the kinesin construct to microtubules. Inhibition of dynein activity had no effect on cell polarisation or adhesion site turnover.

By what mechanism are microtubules guided into adhesion sites? Microtubule motors are not required, since adhesion site targeting by microtubules persisted in cells in which microtubule motor activity was inhibited (Krylyshkina et al., 2002). The results of more recent work suggest that the guidance of microtubule polymerisation into adhesion sites occurs through a mechano-sensing mechanism, involving changes in stress in the actin cytoskeleton. Using alternative strategies to modulate contractile stress locally in living cells, we have found that microtubules specifically invade regions of increased stress, centred on focal adhesions (Kaverina et al., 2002). These findings highlight a stress-dependent feedback

mechanism for guidance that presumably plays an important role in the selection of adhesion sites for targeted modulation via microtubules.

We conclude that microtubules determine cell polarity by influencing, in a spatial way, the turnover of substrate adhesion sites. At the cell front, microtubules can deliver signals to limit the growth of focal adhesions, whereas at the cell rear they promote adhesion sliding and detachment. The age, as well as the local signaling environment of adhesion sites likely plays a synergetic role in this process.

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# Cytoskeletal and lipid domain asymmetry during neutrophil polarization and migration

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The development of cell polarity in response to chemoattractant stimulation in human polymorphonuclear neutrophils (PMNs) is characterized by the rapid conversion from round to polarized morphology with a leading lamellipod at the front and a uropod at the rear. During PMN polarization, the microtubule (MT) array undergoes a dramatic reorientation toward the uropod that is maintained during motility. MTs were excluded from the leading lamella during polarization and motility, but treatment with a myosin light chain kinase inhibitor (ML-7) or the actin-disrupting drug, cytochalasin D, caused an expansion of the MT array and penetration of MTs into the lamellipod. Depolymerization of the MT array prior to stimulation caused some of the cells to lose their polarity by extending two opposing lateral lamellipodia. These multi-polar cells showed altered localization of a leading lamella-specific marker, talin, and a uropod-specific marker, CD44, and dispersal of the endocytic recycling compartment. These results indicate that F-actin and myosin II-dependent forces lead to the development and maintenance of MT asymmetry which may act to reinforce cell polarity through the directed recycling of integrin-containing vesicles to the ERC during PMN migration.

We have also examined the effects of perturbing lipid organization by depleting plasma membrane cholesterol levels. Several receptor-mediated responses, such as integrin upregulation and calcium mobilization, were largely unaffected by cholesterol depletion, indicating that this treatment did not inhibit many aspects of receptor-mediated signal transduction. Interestingly, cholesterol depletion did not prevent initial activation of the GTPase Rac nor an initial burst of actin polymerization, but rather it inhibited prolonged activation of Rac and sustained actin polymerization. These findings support a model in which the plasma membrane is organized into domains that aid in amplifying the chemoattractant gradient and maintaining cell polarization.

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# Dynamic assessment of adhesion molecule functions during lymphocyte migration and initial immune cell-cell interactions

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Leukocyte migration is crucial during the development of the immune system and in the responses to infection, inflammation and tumor rejection. The migratory behavior of leukocytes under physiological and pathological conditions and the extracellular cues and intracellular machinery that control and guide migration have been studied thoroughly during the last years. The cytoskeleton of leukocytes is extremely versatile, bearing characteristic features that enable these cells migrate in conditions of flow through narrow spaces and onto the target tissues. A precise coordination between extracellular stimuli, chemosensory receptors, adhesion and signaling molecules, and the cytoskeleton is fundamental for the migration and/or extravasation of leukocytes (1).

We have investigated the interaction of the adhesion molecules ICAM-3 and PSGL-1 with ERM (ezrin/radixin/moesin) proteins and its role in their membrane positioning in polarized migrating lymphocytes. Protein-protein binding assays demonstrated a $PIP_2$-induced association between ICAM-3 and PSGL-1 and the amino-terminal domain of ERM proteins. This interaction was assessed by dynamic fluorescence videomicroscopy studies of living cells, demonstrating that moesin and ICAM-3 coordinately redistribute on the plasma membrane during lymphocyte migration. Furthermore, overexpression of N-moesin-GFP, which lacks the consensus moesin actin-binding site, caused the subcellular mislocalization of ICAM-3. Point mutation of serine residues in the juxta-membrane region of ICAM-3 and PSGL-1 significantly impaired both ERM binding and polarization of the adhesion molecules. Our results underscore the key role of specific serine residues within the cytoplasmic region of ICAM-3 and PSGL-1 for their ERM-directed positioning at the trailing edge of motile lymphocytes (2).

The interaction of moesin and ezrin with the endothelial cell adhesion molecules VCAM-1 and ICAM-1 has been assessed during leukocyte adhesion and transendothelial migration (TEM). VCAM-1 interacted directly with moesin and ezrin in vitro, and all these molecules colocalized at the apical surface of endothelium. Dynamic assessment of this interaction in living cells showed that both, VCAM-1 and moesin, were involved in lymphoblast adhesion and spreading on the endothelium, whereas only moesin participated in TEM, following the same distribution pattern than ICAM-1. During leukocyte adhesion in static or under flow conditions, VCAM-1, ICAM-1, and activated moesin and ezrin clustered in an endothelial actin-rich docking structure, that anchored and partially embraced the leukocyte, containing other cytoskeletal components such as alpha-actinin, vinculin, and VASP. Phosphoinositides and the Rho/p160 ROCK pathway, which participate in the activation of ERM proteins, were involved in the generation and maintenance of the anchoring structure. These results provide the first characterization of an endothelial docking structure that plays a key role in the firm adhesion of leukocytes to the endothelium during inflammation (3).

Immune cellular interactions occur in sequential stages that involve dramatic changes in cell polarity and dynamic redistribution of cell membrane receptors (4). Antigen-independent adhesive interactions between T lymphocytes and antigen presenting cells (APCs) are critical for the scanning of specific antigen on the APC surface, and the initiation of the immune response. We demonstrate through dynamic studies, that the intercellular adhesion molecule (ICAM)-3 was specifically clustered at the surface area of T lymphocytes that initiates the contact with APCs. ICAM-3 plays a role in T cell-APC conjugate formation prior to antigen recognition, and in early intracellular signaling and cytoskeletal rearrangement of the T cell. Our data indicate that ICAM-3 has a central role in the initial scanning of APC surface by T cells, and therefore, in the generation of the immune response (5).

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# TCR triggering on the move: Diversity of T cell interactions with antigen-presenting cells

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Polarized T cells are mobile cells optimized for migration, receptor scanning, and signaling. In contact with antigen presenting cells (APC), polarized T cells can develop a spectrum of biophysical interaction modes ranging from adhesive sticking to dynamic crawling. Both, static and dynamic contacts support sustained triggering of the T cell receptor (TCR) leading to signal induction, T blast formation, and proliferation. In dynamic interactions, T cells crawl across the surface of antigen-presenting dendritic cells (DC) at speeds of 2-6 μm/min and simultaneously establish an asymmetric tight yet mobile junction plane, representing a dynamic immunological synapse. In dynamic synapses three functional compartments of the polarized T cell are in close contact with the DC surface, i.e. leading edge, cell body and uropod. Through its mobility, the asymmetric junction is topographically suited for receptor scanning and engagement at the leading edge, retrograde receptor movement along the junction, and exit from the uropod. These findings suggest novel concepts on scanning encounters between T cells and APC that include the simultaneous engagement of T cell leading edge and uropod and implicate a serial receptor triggering mode in cell-cell recognition.

## Session 5: Polarized immune cell-cell interactions
## Chair: Abraham Kupfer

# A role for the small GTP-binding protein ARF6 in focal membrane delivery during phagocytosis

Philippe Chavrier

Phagocytosis is a universal cell function, which exploits a mostly conserved cell machinery to couple the binding (in a receptor-dependent way) of particulate material over 0.5 μm in diameter to its internalization [1]. A trademark of phagocytosis is the exquisitely localized actin polymerization that takes place underneath the phagocytic target. During the past years, it has become clear that Rho GTPases control the cytoskeletal rearrangements during particle uptake in mammalian professional phagocytes. In the case of phagocytosis of antibody-coated particles by Fc receptors (FcRs), Cdc42 and Rac1 seem to control different steps in the phagocytic process, namely pseudopod emission for Cdc42 and phagosome closure for Rac1 (for review see [2]).

Recent results have also shown that VAMP3-GFP (Vesicle Associated Membrane Protein 3) accumulates at sites of forming phagosomes during FcR-mediated phagocytosis. This suggests that focal exocytosis of endomembranes plays an important role in pseudopodia extension during phagocytosis by compensating for loss of membrane taken up into phagosomes [3]. Interestingly, a role for ARF6 (ADP-ribosylation factor 6) during phagocytosis has been suggested by studies showing that ARF6 mutant forms could block FcR-mediated phagocytosis [4,5]. ARF6 is a small GTP-binding protein involved in the regulation of endocytosis and membrane recycling toward the plasma membrane, as well as the formation of actin-based plasma membrane extensions [6]. Here, we have started to address the function of ARF6 during phagocytosis. Our findings support a role for ARF6 in linking phagocytic receptor ligation to focal delivery of endomembrane at the site of uptake.

We have developed a new assay to measure the accumulation of GTP-bound ARF6 during phagocytosis. We show that the VHS/GAT amino-terminal domains of GGA3 (Golgi-localized, □-ear-containing, ADP-ribosylation factor-binding protein 3) fused with GST, interacts with a constitutively activated form of ARF6, but not with GDP-bound ARF6. We have used the GST pull-down assay to assess the activation of ARF6 during FcR-mediated phagocytosis in mouse macrophages (RAW cell line). Levels of GTP-bound ARF6 were found to peak after 10 min (x5 fold increase) and returned to basal level after 30 min. Interestingly, treatment of macrophages with pervanadate also resulted in a strong activation of ARF6. Altogether, these results show for the first time that ARF6 is rapidly activated at the onset of phagocytosis, and suggest that tyrosine kinases may be required in the signaling cascade connecting FcR engagement to ARF6 activation.

In addition, we observed that a dominant negative mutant of ARF6 (T27N mutation) dramatically affected both FcR- and CR3-mediated phagocytosis (i.e. phagocytosis of complement-coated particles by the integrin-type Complement Receptor CR3). These observations indicate that ARF6 has a general role in phagocytosis, in contrast to Rac1/Cdc42 or RhoA that are specifically required for FcR- or CR-mediated uptake, respectively. The inhibition of phagocytosis in ARF6T27N-expressing RAW cells was correlated with an inhibition of GFP-VAMP3 accumulation underneath the bound particles. Altogether, these results suggest that ARF6 activation is required for polarized delivery of membrane

originating from recycling (VAMP3-positive) compartment, and that ARF6 is a major link between actin and membrane reorganization during phagocytosis.
We are currently investigating the signal transduction cascades that are involved in coupling ARF6 activation to receptor ligation during phagocytosis in macrophages.

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# Early events in T cell activation and immunological synapse formation in the thymus and the periphery

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We have been interested in the role of key cell surface molecules and their proximal signaling components in the early stages of T cell recognition and activation. In order to visualize molecular movements in real time we have developed a 3D video Microscopy system that allows us to follow the movements of several labeled molecules simultaneously along with morphological changes (DIC) and calcium flux (using ratio imaging). We have used this system to show that the characteristic synapse which forms when T cells recognize antigen on another cell is largely driven by the active transport of membrane molecules, including the T cell receptor, from all over the T cell and into the center of the cell:cell interface. This movement is dependent on an intact actin cytoskeleton and myosin motors and requires both TCR and costimulatory molecule signaling (1-3). In another project we have tried to more precisely define the ligand requirements for T cell recognition and synapse formation by labeling each peptide on an antigen presenting cell such that we detect the exact number of ligands at a T:B interface. We find that $CD4^+$ T cells are extremely sensitive to agonist peptide-MHC complexes and are able to stop and flux calcium in response to even a single peptide on another cell. It requires 10-20 peptides at the interface to reach maximal calcium elevation within the T cell and to form a synapse. This remarkable sensitivity to small numbers of peptides is very much dependant on CD4 being present on the T cell, which suggests a novel model of CD4 function (The "psuedodimer" hypothesis).

We have also shown that immature thymocytes form a distinctly different type of 'synapse' with thymic stromal cells during negative selection. In these synapses CD3ζ-GFP congregates in a peripheral ring at the stromal cell interface, never acquiring the stable central accumulation hat is characteristic of peripheral T cells (4). These data indicate that the characteristics of T cell interactions with other cells can be quite different depending on their maturation status.

With respect to T cell receptor - ligand interactions, we have used alanine mutagenesis to show that TCR binding is a two stage process, where the TCR first comes in contact with the MHC helices and then with the peptide. This initial orientation of the TCR by MHC contact will presumably increase the efficiency with which T cells can 'scan' the peptides displayed another cell. It could also be the molecular explanation for the remarkable cross-reactivity that is characteristic of TCR's as the CDR3 loops that primarily contact the bond peptides could fold over the binding groove in a variety of ways (5).

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# The immunological synapse coordinates antigen recognition, signaling and T cells migration

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My long term goal is to understand cell-cell communication in the immune system. A physical interaction between T lymphocytes and antigen presenting cell is required to initiate and sustain an immune response. This specialized cell-cell junction is described as an "immunological synapse" . We have focused our effort on understanding the molecular basis of the immunological synapse formation and function. This problem must be addressed at many levels, from examination of the behavior of single molecules in the synapse to the study of cell-cell interactions in the living animal. In the past year we have made progress in understanding the basic mechanisms that regulate T lymphocyte activation at the level of understanding the molecular interactions in the immunological synapse. We have also been establishing the tools to enable us to address key aspects of the immunological synapse from tracking single T cell receptor molecules to studying the movements and interaction of T cell in intact lymph nodes of living animals. These studies will increase our understanding of the immune response and aid in the development of vaccines and a new generation of immunologically targeted therapeutics.

The immunological synapse has been defined as a specific pattern of receptors in the interface between a T cell and an antigen presenting cell that is correlated with full T cell activation. We have employed novel imaging techniques to visualize the nascent immunological synapse as a ring of T cell antigen receptor interactions surrounding a core region of adhesion molecule interactions. Over a period of minutes this pattern inverts to generate a mature immunological synapse with a central region of T cell receptor interactions surrounded by a ring of adhesion molecules. We have continued to work on this process. First, we discovered that an important second signal for T cells is also received through a regulated manner in the immunological synapse. Specifically, we found that CD28 interactions with its ligand CD80 is focused in the center of the mature immunological synapse. Second, we found that the mature immunological synapse incorporates not only the foreign MHC-peptide complexes, but also is capable of utilizing self-MHC peptide complexes for its stabilization. This is important because it suggests that self-MHC-peptide complexes are having a large role not only in selection of immature T cells, but also in the activation of mature T cells. This result has important implications for autoimmune disease where self-MHC-peptide complexes contribute to full T cell activation. Third, we found that tyrosine phosphorylation of substrates in the immunological synapse is taking place very rapidly. In fact the activation of src family tyrosine kinases is focused in the nascent synapse and the activation of the next kinase in the cascade, ZAP-70, is coming to an end just as the mature synapse if formed. These studies point to new modes of T cell receptor signaling since it is known that antigen receptor signaling must be sustained for several hours before T cells are fully committed to proliferation and activation of the immune response. Finally, we have found that some cell types, notably human cytotoxic T cells, are capable of forming antigen independent synapses in our model system. This is very exciting because we have previously come to the conclusion that the TCR-MHC-peptide interaction was a key ingredient in

synapse formation. The new result suggests that what we previously saw as subtle variations on a single structure actually represents a family of distinct structures with very distinct regulation. We are completing the final experiments on this system and will submit this for publication in the near future.

We are also very excited by a recent collaboration that we have initiated with Arup Chakraborty at the University of California at Berkeley. Dr. Chakraborty has found that the synapse formation process is very amenable to modeling and that what would appear to be a very complex process, may arise only one step from very simple physical processes. It turns out that the predictions of the synapse formation model are well correlated with IL-2 production by T cells, but not with CTL mediated killing, suggesting that these processes differ in dependence on TCR stimulated immunological synapse formation.

We are motivated by our in vitro results to test the key hypotheses in vivo. We are collaborating with Wenbiao Gan in the Skirball Institute to build a two photon confocal microscope. This technology allows 10-fold increase in depth of high resolution imaging in biological tissues. Wenbiao focuses on the brain and we focus on the lymph nodes and spleen where immunological reactions are focused. In the last few weeks we have obtained beautiful images in the mouse brain and will soon embark on an exploration of immunological synapses in the cervical lymph nodes. The goal of these experiments it to specifically identify differences between cell-cell interactions that lead to immune tolerance or immune responses.

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## Supramolecular clusters and alliances of membrane receptors and signaling proteins during T cell antigen recognition

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The molecular and cellular mechanisms of intercellular cellular communication in the immune system are the main research interests of our laboratory. Activation of T cells by antigen-presenting cells (APCs) depends on the complex integration of signals that are delivered by multiple receptors and by their associated protein kinases.

Most receptor proximal activation events in T cells were identified using multivalent anti-receptor antibodies (Abs), eliminating the need to use the more complex APCs. Since the physiological ligands on the membrane of the APC and the activating Abs presumably trigger the same biochemical pathways, it is unclear why the Abs, even at saturating concentrations, fail to trigger some of the physiological T cell responses. To address this issue, we studied at the single cell level the responses of T cells to native ligands. We utilized a digital imaging system and analyzed the three-dimensional (3D) distribution of receptors and intracellular proteins that cluster at the T-APC cell contacts during antigen (Ag) specific interactions. Surprisingly, instead of a uniform oligomerization, these proteins clustered into segregated 3D domains within the cell contacts. The Ag-specific formation of these novel spatially segregated supra-molecular activation clusters (SMACs) as well as their functional significance would be discussed.

# POSTERS

## Lipid rafts in yeast polarity

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Exposure to mating pheromone in haploid *Saccharomyces cerevisiae* cells results in the arrest of the cell cycle, expression of mating-specific genes, and polarized growth towards the mating partner. Proteins involved in signaling, polarization, cell adhesion, and fusion are localized to the tip of the mating cell (shmoo) where fusion will eventually occur. The mechanisms ensuring the correct targeting and retention of these proteins are poorly understood. Our results show that in pheromone-treated cells a reorganization of the plasma membrane involving lipid rafts, results in the segregation of different domains within the plane of the membrane. Thus, proteins localized to tip of shmoo can achieve a polarized distribution, separated from other cell surface proteins. Our results suggest that protein clustering into membrane micro domains is involved in the generation and maintenance of polarity during mating.

## EB1 and APC act downstream of Rho/mDia in a conserved pathway between yeast and mammals

Noemí Cabrera-Poch, Ying Wen, and Gregg G. Gundersen

Microtubules (MT) play an essential role in generating cell polarity. In polarized cells, a subset of MT are stabilized in the lamella. These stable MT may provide cells with cues for polarized vesicle trafficking or migration. Stable MT are capped and contain post-translationally modified tubulin subunits. The best characterized modification is detyrosination, in which the C-terminal Tyr residue from a-tubulin is removed exposing a Glu residue (Glu MT). Lysophosphatidic acid (LPA) has been found to induce the formation of Glu MT in serum starved NIH-3T3 cells in the wounded monolayer system. Rho GTPase and its effector mDia mediate LPA-induced MT stabilization in this system by capping MTs (Cook et al., JCB 1998; Palazzo et al., NCB 2001). In yeast, an analogous process, MT capture, is regulated by Rho GTPases, Bni1 (an mDia homolog) and the downstream components Kar9 and Bim1/Yeb1 (Heil-Chapdelaine et al., JCB 1999; Bloom, NCB 2: E96). We examined whether the mammalian homolog of yeast Bim1/Yeb1,EB1,and APC (Adenomatous polyposis coli protein, a putative functional homolog of Kar9) were also involved in this pathway of MT stabilization. Expression of full length EB1 and APC were sufficient to generate microtubule stabilization. EB1 acted downstream of Rho/mDia since it induced stable MTs in cells coinjected with the Rho inhibitor, C3 toxin, without stimulating the formation of actin stress fibers. A fragment of EB1, corresponding to the APC binding domain, blocked LPA-stimulated formation of stable MTs, whereas another fragment lacking this domain did not. These results show that EB1 and APC function in the Rho/mDia MT stabilization pathway, and suggest that pathways for MT capture in yeast and MT capping in mammalian cells are conserved.

## Myosin motors but not actin comets mediate Golgi-to-ER protein transport

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We have demonstrated that actin filaments are involved in protein transport from the Golgi complex to the endoplasmic reticulum. Here we examined whether myosin motors or actin comets mediate this transport. We used two pharmacological inhibitors of myosin motors with differing specificity: BDM (2,3-butanedione monoxime) and ML7 (1-[5-isoquinoline sulfonyl]-2-methyl piperazine), which inhibit myosin and the phosphorylation of myosin II by the myosin light chain kinase, respectively. Treatment of NRK cells with these reagents delayed the brefeldin A (BFA)-induced retrograde flow of Golgi enzymes to the ER. This was not caused by an alteration in the formation of the BFA-induced tubules emerging from the Golgi complex, although they remained in the cytoplasm longer before fusing with the ER membranes. In addition, the Shiga toxin fragment B transport from the Golgi complex to the ER was also altered by BDM and ML7. This alteration was not attributable to the depletion of intracellular calcium stores or the activation of Rho kinase. Neither the reassembly of the Golgi complex after the withdrawal of BFA nor VSV-G transport from the ER to the Golgi was altered in cells treated with BDM or ML7. Finally, transport carriers containing Shiga toxin did not associate with actin comets in response to PIP5K overexpression. These results indicate that: (1) Golgi-to-ER but not ER-to-Golgi protein transport requires myosin function, implicating at least myosin II and (2) actin comets do not appear to be involved in retrograde transport.

# a4b1 integrin engagement inhibits a5b1-induced stress fiber and focal contact formation via downregulation of RhoA and induces melanoma cell migration

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Cellular interactions with the extracellular matrix (ECM) are essential for cytoskeleton reorganization, cell migration, survival and differentiation. We have studied the cytoskeletal response upon melanoma cell interaction with two different domains of fibronectin (Fn), a major component of the ECM. Using recombinant fragments encompassing the RGD-containining cell binding domain (FNIII7-10) or the Hep III domain (FNIII4-5), which are ligands for a5b1 and a4b1 integrins respectively, we have found that melanoma cells formed stress fibers and focal contacts upon adhesion to FNIII7-10. Co-inmobilization of FNIII4-5 with FNIII7-10 resulted in inhibition of stress fibers and focal adhesions as cells became smaller and extended multidirectional cytoplasmic protrusions. This effect was mediated by a4 integrin since point mutations affecting the a4-binding site in FNIII4-5 completely reverted the inhibition. Treatment with 1 mM LPA, an activator of the small GTPase RhoA or transient transfection with constitutively active RhoA resulted in spreading and formation of stress fibers upon adhesion to FNIII7-10+FNIII4-5 fragments. Affinity binding assays revealed that a4 ligation led to RhoA inactivation. Since lack of stress fibers is associated with cell migration, we monitored cell movement using video microscopy and found that cells on FNIII7-10+FNIII4-5 displayed significant random migration while cells on FNIII7-10 did not move. This was further confirmed in wound-healing assays, where addition of FNIII4-5 resulted in almost complete closure of the wound, while addition of FNIII7-10 did not affect cell motility and the wound remained open. These data indicate that a4 integrin engagement interferes with the Rho signalling pathway activated by a5/ligand interaction and this leads to stimulation of melanoma cell migration.

# Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization

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Cell migration is an important process for leukocyte function. In order to migrate, cells must adquire spatial and functional polarity. T lymphocytes develop two opposite poles during polarization process, the leading edge located at the advance front, and the uropod located at the rear. Evidence has emphasised the importance of glycosphingolipids and cholesterol-enriched membrane domains - termed rafts- in mediating T cell activation. More recently, membrane rafts have been also implicated in the assymetric redistribution of proteins observed in chemoattractant stimulated T cells.

We reported here that raft-associated membrane proteins and lipids are asymmetrically distributed in T cells with a migrating phenotype. We found that polarized T lymphocytes segregate leading edge and uropod markers into two different raft types, the L-rafts and the U-rafts, which differ in lipid and protein composition; L-rafts are enriched in GM3 and chemoattractant receptors such as CXCR4, whereas U-rafts are enriched in GM1 and cell adhesion receptors such as CD44 and ICAM-1 and -3. The redistribution of both L- and U-rafts is susceptible to actin depolymerizing drugs, thus suggesting that it requires an intact actin cytoskeleton. Conversely, disruption of the microtuble cytoskeleton does not affect asymmetric raft distribution in polarized T cells.

Our data suggest that raft partitioning is a major determinant for asymmetric protein distribution in polarized T cells, as ectopically expressed raft-associated proteins are redistributed during cell polarization. Conversely, modification of these proteins in such a way that they do not associate with rafts inhibits their asymmetric distribution. On the other hand, disruption of L- and U-rafts by chemical depletion of membrane cholesterol impedes cell polarization, chemotaxis and recruitment of bystander cells; replenishment of membrane cholesterol restores all these functions.

In summary, we propose that membrane protein segregation not only between raft and nonraft membrane domains but also between distinct rafts subdomains may be an organizational principle that mediates redistribution of specialized molecules needed for T cell migration.

## Lipid rafts mediate biosynthetic transport to the T lymphocyte uropod subdomain and are necessary for uropod integrity and function

Millán, J., Montoya, M.C., Sancho, D., Sanchez-Madrid, F.and Alonso, M.A.

Polarized migrating T cells possess 2 poles, the uropod protrusion at the rear and the leading edge at the front, with specific protein composition and function. The influenza virus hemagglutinin (HA) is a prototypical molecule that uses lipid rafts for biosynthetic transport to the apical surface in polarized epithelial Madin-Darby canine kidney (MDCK) cells. In this study, HA was used as a tool to investigate the role of lipid rafts in vectorial protein traffic in polarized T lymphocytes. Results show that newly synthesized HA becomes selectively targeted to the uropod subdomain in polarized T lymphoblasts. HA incorporates into raft soon after biosynthesis, suggesting the delivery of HA to the uropod occurs through a pathway of transport reminiscent of that used for its specific targeting to the apical surface. Polarized adhesion molecules, intracellular adhesion molecule 3 (ICAM-3), CD44, CD43, 3 endogenous uropod markers were also detected in surface rafts. Cholesterol, the major component of lipid rafts, was predominantly located in the uropod. Disruption of lipid raft integrity by cholesterol sequestration produced unclustering of ICAM-3 and the loss of uropodia, and severely impaired processes that require a polarized phenotype such intracellular aggregation and cell migration. Collectively, these results indicate that lipid rafts constitute a route for specific targeting of proteins to the uropod and that rafts are essential for the generation, maintenance and functionality of T-cell anteroposterior polarity.

## Essential contribution of CD3γ to T-cell receptor-mediated phosphorylation, adhesion and apoptosis in human mature T lymphocytes

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CD3 proteins may have redundant as well as specific contributions to the intracellular propagation and final effector responses of TCR-mediated signals at different checkpoints during T-cell differentiation. We report here on the participation of CD3γ in the activation and effector function of human mature T lymphocytes at the antigen recognition checkpoint. Following TCR/CD3 engagement of human CD3γ-deficient T cell lines, and despite their lower TCR/CD3 surface levels compared to normal controls, mature T-cell responses such as the regulation of expression of several cell surface molecules, were either normal or only slightly delayed. In contrast, an early extinction of protein tyrosine phosphorylation was observed. Other physiological responses like the specific adhesion and concomitant cell polarization on ICAM-1-coated dishes were selectively defective, and activation-induced cell death was increased. Our data indicate that CD3γ contributes essential specialized signaling functions to certain mature T cell responses. Failure to generate appropriate interactions may abort cytoskeleton reorganization and initiate an apoptotic response.

## Pyk2 regulatory function on MTOC translocation and T cell response through an adapter role for Fyn during superantigen stimulation

David Sancho

The relocation of kinases during binding of a T cell to an appropriate antigen-presenting cell (APC) is essential for lymphocyte activation. Immunofluorescence and live fluorescence microscopy showed that the proline-rich tyrosine kinase-2 (PYK-2) is rapidly translocated to the cell-cell contact area upon T cell specific recognition of superantigen pulsed APCs. The activation with anti-CD3-coated latex microspheres was sufficient for Pyk2 reorientation, and CD28 co-engagement gave an additive effect. The absence of functional Lck, but not of ZAP-70, prevented TCR-mediated Pyk2 rearrangement but not its activation. Moreover, analysis of chimeric molecules of CD8 containing in its cytoplasmic region one immunoreceptor tyrosine-based activation motif (ITAM) from TCR-zb or CD3e, or a non-functional ITAMe demonstrated that lck-mediated Pyk2 translocation and activation required the presence of at least one intact ITAM. In addition, Pyk2 colocalized with Fyn at the MTOC of T cells. Overexpression of a kinase-dead mutant of Pyk2H did not affect TCR-induced Pyk2/MTOC translocation, suggesting that Pyk2 reorientation was independent of its activation. In contrast, a mutant Pyk2H-Y402F, unable to bind Fyn, significantly inhibited MTOC complex translocation and IL-2 production, but not the induction of CD69 expression in response to superantigen stimulation. Therefore, Pyk2 plays a critical role for coupling Fyn to the MTOC complex, which is essential for the early relocation of the MTOC and its associated signaling complex, necessary for the amplification and maintenance of the T cell response.

## Rho4p, a new GTPase involved in cell polarity processes in *S. pombe*

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The Rho family of GTPases are present in all eukaryotic cells, from yeast to mammals and their role as key regulators in the signalling pathways that control actin organization and morphogenetic processes is well known.

In yeast, Rho GTPases are implicated in cell polarity processes and cell wall biosynthesis. The yeast *Schizosaccharomyces pombe* have six rho GTPases, Rho1-5 and Cdc42. It is known that Rho1 and Rho2 are key proteins in the construction of the cell wall, an essential structure that in *S. pombe* is composed of glucans (beta and alfa) and mannoproteins. Here we will describe the characterization of Rho4, another important Rho GTPase for fission yeast morphogenesis. Overexpression of rho4+ produces drastic lisis that can be rescued by adding sorbitol, suggesting a possible role of Rho4p in cell wall dynamics. Deletion of rho4+ produce viable cells with defects in cytokinesis at high temperature. In addition, Rho4p is a protein with steady-state levels throughout the cell cycle and Rho4-GFP localizes to the septum but not to other growth sites in the cell. All these data suggest a role of Rho4p in polarity processes in *S. pombe* that will be discussed.

## A juxta-membrane amino acid sequence of P-selectin glycoprotein ligand-1 is involved in moesin binding and ERM-directed targeting at the trailing edge of migrating lymphocytes

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P-selectin glycoprotein ligand 1 (PSGL-1) is an adhesion receptor localized on the tips of microvilli that is involved in the rolling of neutrophils on activated endothelium. We found that PSGL-1 was concentrated at the uropod of chemokine-stimulated lymphoid cells. Dynamic fluorescence videomicroscopy analyses of migrating lymphocytes demonstrated that PSGL-1 and moesin redistributed towards the cellular uropod at the trailing edge of these cells, where activated ezrin/radixin/moesin (ERM) proteins were located. An eighteen amino acid sequence in the juxta-membrane region of the PSGL-1 cytoplasmic tail was found to be critical for uropod targeting and moesin binding. Substitution of Ser336, Ser348, and the basic cluster R337K338 by alanines within this region significantly impaired both moesin binding and PSGL-1 polarization. These results underline the role of moesin in the subcellular redistribution of PSGL-1 in lymphoid cells.

# Temporal and spatial regulation of Cdc24p, a GEF for the small GTPase Cdc42p in yeast

Yukiko Shimada, Marie-Pierre Gulli and Matthias Peter

The actin cytoskeleton is a highly dynamic structure that is required for many biological processes including establishment of cell polarity. It is thought that local activation of Rho family GTPases regulates cell polarity, but little is known about the spatial and temporal regulation of this process.

In the budding yeast *Saccharomyces cerevisiae*, activation of the GTPase Cdc42p by its guanine nucleotide exchange factor (GEF) Cdc24p is a prerequisite for orientation of the actin cytoskeleton (1). Since Cdc24p is recruited to the incipient bud site (2), we investigated the mechanism that regulates Cdc24p during budding. We revealed an import role for the GTPase Bud1p in the recruitment of Cdc24p to the plasma membrane. Indeed, a Cdc24p mutant that is unable to interact with Bud1p fails to localize to the plasma membrane. Expression of artificially membrane-targeted Cdc24p (myr-Cdc24p) does not interfere with cell growth, suggesting that membrane recruitment is not sufficient for its activation. However, co-expression of Bud1p-GTP renders myr-Cdc24p highly toxic, implying that Bud1p may not only recruit Cdc24p to the plasma membrane but also activate it in vivo. Finally, we uncovered an auto-inhibitory effect of the Cdc24p C-terminus on its in vivo activity. This domain is also the binding site for Bem1p, an effector of Cdc42p. These results suggest a role for Bem1p in stabilizing the active Cdc24p at the site of polarized growth.

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# Regulation of cell spreading and motility by a novel Trp channel-related kinase

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Actomyosin contractility is regulated by Rho family GTPases in combination with changes in cytosolic free $Ca^{2+}$ and fundamental to diverse phenomena as neurite retraction, the formation of focal adhesions, cell spreading and - migration. In previous studies, we identified a myosin II heavy chain (MHC) kinase activity that is activated by $Ca^{2+}$-mobilizing receptor agonists and implicated in actomyosin disassembly and cell spreading. Activation of this MHC kinase apears to be dependent on $Ca^{2+}$-influx and regulated by the Rac1 GTPase (van Leeuwen, 1999). To date, the role of MHC phosphorylation in vertebrate cells is not well understood, and the responsible kinases have not been identified. In contrast, in the lower eukaryote *Dictyostelium*, MHC kinases are key regulators of actomyosin disassembly and cell motility.

We have identified a novel vertebrate kinase related to *Dictyostelium* MHC kinases. Surprisingly, upstream of the kinase domain, the protein is highly similar to a family of receptor-stimulated $Ca^{2+}$ channels, known as 'Transient receptor potential' (Trp) channels. Mouse and human homologs of this chimeric protein have recently been identified as TRP-PLIK (Runnels, 2001) and LTRPC7 (Nadler, 2001), but its function remains elusive. Here we show that TRP-PLIK functions as a receptor-operated calcium channel that associates with myosin II and regulates the actomyosin cytoskeleton. Evidence is provided to show that TRP-PLIK may function as a vertebrate myosin heavy chain kinase.

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## Peritoneal dialysis induces an epithelial-mesenchymal transition of mesothelial cells

María Yáñez-Mó, Enrique Lara-Pezzi, Marta Ramírez, Carmen Domínguez-Jiménez, Francisco Sánchez-Madrid, and Manuel López-Cabrera

During continuous ambulatory peritoneal dialysis, the peritoneum is directly exposed to bioincompatible dialysis fluids, which cause progressive denudation of mesothelial cells, tissue fibrosis and ultrafiltration failure. However, the patho-physiology of the process has not been elucidated so far. We demonstrate that peritoneal mesothelial cells undergo an epithelial to mesenchymal transition during CAPD and acquire a migratory phenotype. Most of these changes are already evident at the early stages of the transdifferentiation, soon after CAPD is initiated. In vitro analysis point to wound repair, profibrotic and proinflammatory cytokines as crucial initiating factors of mesothelial cell transdifferentiation. Immunohystochemical studies of peritoneal biopsies demonstrated the presence of cytokeratin-expressing fibroblast-like cells entrapped in the stroma, suggesting that these cells raised from local conversion of mesothelial cells. Our results indicate an active role of mesothelial cells in the structural and functional alteration of the peritoneum during CAPD and suppose a new concept in the pathology of ultrafiltration failure. Our data also unveil a series of potential markers and targets in the design of new dialysis solutions and follow up of the patients.

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