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# Rab8 and Rab8-interacting proteins as players in cell polarization

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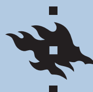
Helsinki 2007



# Rab8 and Rab8-interacting proteins as players in cell polarization

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*Academic dissertation*

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# ORIGINAL PUBLICATIONS

**I.** **Hattula, K**. and Peränen, J. (2000). FIP-2, a coiled-coil protein, links Huntingtin to Rab8 and modulates cellular morphogenesis. *Curr. Biol.,* **10**:1603-1606.

**II.** **Hattula, K**., Furuhjelm, J., Arffman, A., and Peränen J. (2002). A Rab8-specific GDP/GTP exchange factor is involved in actin remodeling and polarized membrane transport. *Mol. Biol.Cell,* **13**:3268-3280.

**III.** **Hattula, K**., Furuhjelm, J., Tikkanen, J., Tanhuanpää, K., Laakkonen, P., and Peränen J. (2006). Characterization of the Rab8-specific membrane traffic route linked to protrusion formation. *J. Cell Sci.*, **119**: 4866-4877.

# ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine diphosphate |
| ADPKD | autosomal dominant polycystic kidney disease |
| AJ | adherence junction |
| ATP | adenosine triphosphate |
| BFA | brefeldin A |
| CCV | clathrin coated vesicle |
| CCP | clathrin coated pit |
| CDS | coding sequence |
| cDNA | complementary DNA |
| COP | coatamer protein |
| C-terminal | carboxy-terminal |
| CTxB | cholera toxin B |
| cyt D | cytochalasin D |
| DN | dominant negative |
| DNA | deoxyribonucleic acid |
| DH domain | Dbl-homology domain |
| ECM | extracellular matrix |
| EE | early endosome |
| EGFP | enhanced green fluorescent protein |
| ER | endoplasmic reticulum |
| ERC | endocytic recycling compartment |
| EST | expressed sequence tags |
| F-actin | filamentous actin |
| G-actin | globular actin, actin monomer |
| GAP | GTPase activating protein |
| GCK | germinal center kinase |
| GDI | guanosine nucleotide dissociation inhibitor |

| | |
|---|---|
| GDF | GDI displacement factor |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GGTase | geranyl-geranyl transferase |
| GST | glutathione-S-transferase |
| GTP | guanosine triphosphate |
| HGF | hepatocyte growth factor |
| His | histidine |
| MAP | mitogen-activated protein |
| MDCK | Madine-Darby canine kidney cell line |
| MHCI | major histocompatibility complex class I |
| mRNA | messenger RNA |
| MTOC | microtubule organizing center, e.g. the centrosome |
| NRP | NEMO related protein |
| NSF | N-ethylmeleimide sensitive factor |
| N-terminal | amino-terminal |
| PA | phosphoric acid |
| PH domain | plextrin homology domain |
| PI-3 kinase | phosphoinositol-3 kinase |
| PIP2 | phosphatidylinositol (4,5)-diphosphate |
| PIP5K | phosphoinositol (4) phosphate 5-kinase |
| PKC | protein kinase C |
| PLD | phospholipase D |
| PM | plasma membrane |
| REP | Rab escort protein |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RTP | rhodopsin transport carrier |
| SH2 | Src homology type 2 domain |
| SH3 | Src homology type 3 domain |
| siRNA | short interfering RNA |
| SNAP | soluble NSF attachment protein |
| SNARE | SNAP receptor |
| Tfn | transferrin |
| Tfn-R | transferrin receptor |
| TGN | trans-Golgi network |
| TJ | tight junction |
| TNF-α | tumor necrosis factor-α |
| VSVG protein | vesicular stomatitis virus G protein |
| wt | wild type |
| ZO | zonula occludens |

## ABSTRACT

During division, differentiation, and migration cells undergo polarization by reorganizing internal and external components, such as actin, microtubules, and adhesion receptors. In moving cells Rho-family small GTPases control the dynamic formation of lamellipodia, filopodia, and focal adhesions. However, the role of membrane traffic in modulating cell polarity and cell migration is controversial. Rab small GTPases control distinct steps of vesicle transport, and are likely candidates in controlling targeting of vesicles. Rab8 is a small GTPase that has been shown to regulate cell morphogenesis by reorganizing both actin and microtubules. It induces the formation of new surface extensions and has an important role in directed membrane transport to cell surfaces. This raises the possibility that Rab8 controls a membrane trafficking route that participates in the establishment of cell polarity.

I set out to find novel interactors of the small GTPase Rab8 in order to find out more about its function in the cell. One of the proteins found, Rabin8, interacts with Rab8 specifically in the GDP-bound form. GDP release and GTP exchange on Rab8, but not on Rab3A or Rab5, is stimulated by Rabin8, indicating that this protein is a Rab8-specific GEF. On a cellular level, we have observed that Rabin8 gets recruited onto vesicles where presumably, it meets and activates its target, Rab8. In addition, we have observed that the Rabin8 protein localizes to cortical actin. Expression of Rabin8 in cells result in both remodeling of actin and in formation of polarized cell surface domains.

I also show that FIP-2, a tumor necrosis factor-α (TNF-α)-inducible protein, interacts with Rab8 specifically in its GTP-bound form. Rab8 binds an amino-terminal region of FIP-2 and the Huntingtin protein binds a carboxy-terminal region on FIP-2. Co-expression of FIP-2 and Huntingtin enhanced the recruitment of Huntingtin to Rab8-positive vesicular structures, and FIP-2 promoted cell polarization in a similar way to Rab8. I furthermore present a second protein interacting with Rab8 specifically in its GTP-bound form. JFC1, a member of the synaptogamin-like protein (Slp) family, is also known to interact with Rab27a in a nucleotide specific manner. What's more, both Rab8 and Rab27a participate in the actin-dependent movement of melanosomes, suggesting that they may functionally overlap. JFC1 co-localizes with endogenous Rab8 on tubular and vesicular structures and is, we believe, involved in controlling Rab8 membrane dynamics.

I show that both Rab8-depletion by siRNA transfection and expression of the dominant negative Rab8 (T22N) promote cell-cell adhesion and the formation of symmetric cells. In contrast, expression of the dominant active Rab8 (Q67L) decreases contact inhibition and promotes the formation of asymmetric cells with protrusions. I demonstrate that Rab8 is associated with macropinosomes generated at ruffling areas of the membrane. These macropinosomes fuse with or transform into tubules that move toward the cell center, from where they are recycled back to the leading edge to participate in protrusion formation. I furthermore show that the biogenesis of these Rab8-tubules is dependent on both microtubules and actin dynamics.

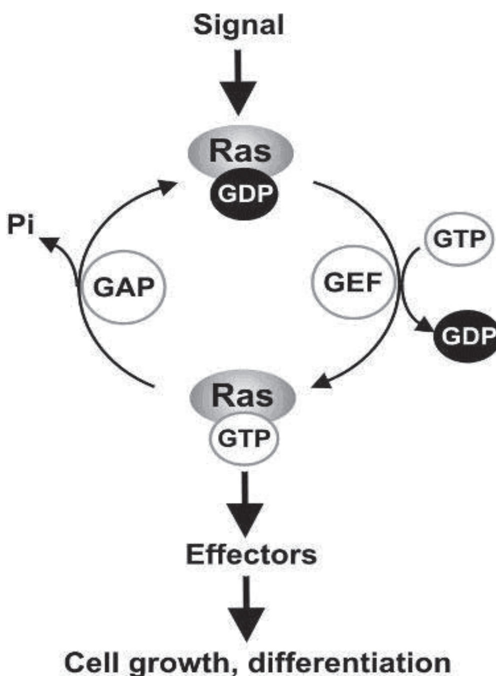
The Rab8-specific membrane route contained several markers known to be internalized and recycled (β1 integrin, transferrin, transferrin receptor, cholera toxin B subunit (CTxB), and major histocompatibility complex class I protein (MHCI). Rab8 localization overlaps with both Rab11 and Arf6, and is functionally linked to Arf6. I propose that Arf6 and Rab8 together define a recycling pathway that mediates protrusion formation.

# 1. INTRODUCTION

## 1.1 Small GTP-binding proteins

The Ras super-family of small GTP-binding proteins is a large group of proteins that are found in eukaryotes from yeast to human, ranging in size between 20-40 kDa. They can structurally and functionally be divided into at least five subfamilies: Ras, Rho, Rab, Sar/Arf, and Ran. (Zerial and Huber 1995, Hall 2000). These proteins function as molecular switches by cycling between a GTP-bound (active) and a GDP-bound (inactive) conformation (Bourne *et al.* 1990). The Ras family members mainly regulate gene expression, controlling cell proliferation and differentiation. The Rho family members mainly regulate cytoskeletal reorganization but also have an effect on gene expression. The Rab family is the largest subfamily and its members regulate vesicle trafficking. The Sar/Arf family control vesicle budding and the Ran family regulate nuclear transport as well as microtubule organization during mitosis.

All small GTPases except Sar1 and Ran have a sequence that undergoes post-translational modification with lipid. The lipid modification is essential for their binding to membranes and for their biological functions (Magee *et al.* 1992, Zhang and Casey 1996). Arfs are N-terminally myristoylated whereas Ras, Rho and Rab proteins are prenylated at one or two cystein residues at the C-terminus. All GTPases have consensus amino acid sequences for interaction with GDP/GTP and for GTPase activity, and they all have regions for interaction with downstream effectors (Bourne *et al.* 1991, Valencia *et al.* 1991). Based on mutations originally found in Ras, it has been possible to produce constructs locking different GTPases either in their GTP or GDP bound forms. These constructs have been very useful in elucidating the functions of individual small GTPases.




The GTPase cycle is a tightly controlled process where release of bound GDP leads to binding of GTP and a conformational switch, which allows the GTPase to bind downstream effectors and activate them (see Fig. 1). De-activation of the GTPase occurs by hydrolysis of the bound GTP to GDP and inorganic phosphate. Regulation of this cycle is controlled by three groups of molecules: guanine nucleotide exchange factors (GEFs), GTPase activating proteins

**Fig. 1. The GTPase cycle and its regulators.** GTPases can function as molecular switches in a variety of biological processes as a result of the conformational change upon GTP binding or hydrolysis. GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; Pi, inorganic phosphate.

(GAPs), and in the case of Rho and Rab proteins GDP dissociation inhibitors (GDIs). GEFs interact with the GDP bound form of the GTPase to facilitate the release of the bound GDP and the subsequent binding of GTP, thereby activating the protein. Once GTP is bound the GTPase is free to interact with its downstream effectors(s). De-activation is managed by GAPs, which interact with the GTP form of the GTPase facilitating hydrolysis of the bound GTP to GDP. The exact mechanism for this event is not known, however, it may be brought about by the GAP speeding up the intrinsic GTPase activity by stabilizing the optimal conformation or by the GAP contributing enzymatic activity. The third type of regulator is the GDI; this molecule specifically binds to the lipid-modified GDP-bound form of the GTPase keeping it in the inactive conformation both by inhibiting the intrinsic exchange activity and by preventing activation by a GEF. The GDI also has the ability to extract the GTPase from the membrane and to then keep it as a soluble complex in the cytoplasm. This activity is very important for the functions of Rho and Rab proteins, which need to couple their activation/deactivation cycles with a cycle of localization on and off certain places on membranes in the cell. Tightly controlled GTPase cycles enable the GTPases to function not only as a molecular switches but also as a biological timers, controlling the exact time and position of a certain biological event. Each of the small GTPases may have the potential to interact with more than one downstream effector, which would allow it to transmit different downstream signals and different cellular effects. (Hall 2000).

### *1.1.1 The Ras family*

Ras proteins, constituting a family of 13 members, regulate a wide range of cell functions such as proliferation, differentiation, morphology, and apoptosis (Feramisco *et al.* 1984, Bar-Sagi and Feramisco 1985, Kauffmann-Zeh *et al.* 1997). When activated by point mutations, the three most widely studied members of this family; Ha-Ras, K-Ras, and N-Ras, have been found able to transform mammalian cells (Brown *et al.* 1984, Capon *et al.* 1983, Feramisco *et al.* 1984, Stacey and Kung 1984). Generally when Ras proteins are mentioned it is referring to these three members. Mutation of Ras genes or their regulator genes causes human cancer. As much as 20-30% of all cancers may be directly or indirectly caused by mutations of Ras molecules (Barbacid 1987, Bos 1989).

Ras proteins are permanently located on membranes (Choy *et al.* 1999). Their activity is triggered by GEFs, mainly downstream of signals mediated by membrane receptors with their own or an associated tyrosine-kinase activity. Additionally, second messengers such as calcium and diacylglycerol can also activate Ras in some cell types (Hall 2000). The tyrosine kinase motif associated with a membrane receptor attracts a Ras GEF that in turn activates the Ras GTPase. Activated Ras is then free to interact with its downstream effectors. The best studied Ras effector is Raf kinase. In the MAP kinase cascade Raf phosphorylates and activates MEK (a MAP kinase kinase) that in turn phosphorylates and activates ERK (a MAP kinase). ERK translocates to the nucleus where it phosphorylates and activates various transcription factors. Another well known Ras effector is the catalytic subunit of PI3K, mainly leading to protection against apoptosis. Through a distinct pathway PI3K can also activate Rac leading to the

formation of cell surface protrusions. Finally there is the RasGEF family, the prototype being RalGDS, which activates Ral. Signaling complexes are in this way assembled at the original site of action, leading to changes in gene expression in the cell and affecting processes like cell growth, differentiation and morphology. The biological outcome of Ras signaling is very much dependent on the cell type and other signaling events in the cell. Important and poorly understood factors are the intensity of the signal produced as well as its duration. (Takai *et al.* 2001, Hall 2000).

### *1.1.2 The Rho family*

The function of Rho proteins was first demonstrated in yeast where they were shown to be involved in the budding process, presumably through reorganization of the actin cytoskeleton (Johnson and Pringle 1990, Yamochi *et al.* 1994). There are believed to be 20 members in mammals, 18 so far described (Schultz *et al.* 1998, Govek *et al.* 2005). Rho proteins are key regulators of the actin cytoskeleton in all eukaryotic cells and have additional roles in microtubule cytoskeleton reorganization (Wittmann and Waterman-Storer 2001), gene expression (Coso *et al.* 1995, Hill *et al.* 1995, Perona *et al.* 1997) and membrane transport processes (Brown *et al.* 1998, Komuro *et al.* 1996, Lamaze *et al.* 1996). The three most intensively studied Rho proteins; RhoA, Rac1 and Cdc42, have their own niches in the regulation of the actin cytoskeleton. RhoA controls the formation of stress fibers (bundles of actin filaments that traverse the cell) and focal adhesions (Miura *et al.*1993, Ridley and Hall 1992). Rac1 regulates the formation of lamellipodia (thin protrusive actin sheets at the leading edge of a migrating cell) and membrane ruffles (Ridley *et al.* 1992). Finally, Cdc42 controls the formation of filopodia (fingerlike protrusions that contain tight bundles of long actin filaments in the direction of the protrusion) (Kozma *et al.* 1995, Nobes and Hall 1995). Additionally, all three induce the assembly of multi-molecular focal complexes at the plasma membrane of fibroblasts (Nobes and Hall 1995). Since these are central features of cell migration it is not surprising to find that RhoA, Rac1 and Cdc42 play a crucial role in controlling cell migration. Cdc42 is required also for another important aspect of cell migration: the establishment of cell polarity (Etienne-Manneville and Hall 2002).

Just as for Ras proteins, the Rho GTPase activity is regulated by GEFs and GAPs. However, Rho proteins are additionally regulated by a third protein called GDI. The Rho protein is kept as an inactive complex with the GDI in the cytosol and has to be released from the GDI before it can be activated by a GEF. The mechanism for this release is largely unknown but ERM (ezrin/radixin/moesin) proteins may be somehow involved (Takahashi *et al.* 1997). There are ~60 Rho-family GEFs in humans, including several oncogenes (Schmidt and Hall 2002, Schultz *et al.* 1998). They all contain a Dbl-homology (DH) domain that is required for GEF activity (Hart *et al.* 1994). Most Rho GEFs also contain a plextrin-homology (PH) domain, adjacent and C-terminal to the DH domain. This domain is thought to be involved in proper localization in the cell, probably through an interaction with $PIP_2$ (Zheng *et al.* 1996, Rameh *et al.* 1997). Additionally, many Rho GEFs have other functional domains including src homology (SH2 and SH3) domains, serine/threonine or tyrosine kinase domains, suggesting that they may have functions other than assisting the guanine nucleotide exchange on Rho-

family proteins (Bishop and Hall 2000, Schmidt and Hall 2002). So far, 40 GAPs have been cloned (see Moon and Zheng 2003) and three different GDIs (Takai *et al.* 2001, Hall 2000).

Around 40 downstream effectors of mammalian Rho protein have been found (Bishop and Hall 2000, Raftopoulou and Hall 2004). One of these, p160ROCK (also called ROK$\alpha$ or Rho kinase) is implicated in several pathways involved in actin rearrangement. It also co-operates with another Rho effector, p140mDia, in promoting stress fibers (Takai *et al*. 2001). The major targets for Rac and Cdc42, in mediating actin polymerization in protrusions, are the WASP/WAVE family proteins (Ridley *et al*. 2003). Rac stimulates lamellipodial extension by activating WAVE proteins (Cory and Ridley 2002), and Cdc42 binds to WASP proteins (Ridley *et al*. 2003). Interestingly, it has been reported that WAVE/WASP proteins bind to Rho-family GAPs and GEFs, potentially creating positive or negative feed-back loops to regulate the extent of actin polymerization (Ridley *et al*. 2003).

### *1.1.3 The Ran family*

There is only one Ran protein in many cell types (including human) or then two or more closely related Ran genes in other organisms (e.g. *S. cerevisiae*) (Moore 1998). Ran plays a central role in both nuclear import and export (Moore and Blobel 1993, Ohno *et al.* 1998), and has also been implicated in microtubule organization during the M-phase of the cell cycle (Carazo-Salas *et al.* 1999, Kahana and Cleveland 1999). Its regulators are asymmetrically distributed between the nucleus and the cytoplasm. This leads to Ran-GTP being found exclusively in the nucleus where the Ran-GEF, RCC1 (Bischoff and Ponstingl 1991), is localized and Ran-GDP found only in the cytoplasm where it's GAP, Ran GAP1 (Bischoff *et al.* 1994), is located. The gradient of GDP and GTP-bound Ran plays a key role in the directionality of transport between nucleus and cytoplasm (Izaurralde *et al.* 1997). Ran unlike other small GTPases does not bind to membranes inside the cell and does not need lipids for its activity (Rush *et al.* 1996).

### *1.1.4 The Sar/Arf family*

Sar1 from *S. cerevisiae* was the first member of this subfamily of small GTPases to be isolated. It was shown to function in the assembly of COP II-coated vesicles in membrane transport from the ER to the Golgi apparatus (Nakano and Muramatsu 1989). Two Sar1 proteins (Sar1a and Sar1b) (Kuge *et al.* 1994) and six Arf proteins (Arf1-6) have been found in mammals (Moss and Vaughan 1995). Arf proteins act to regulate membrane traffic and organelle structure (Chavrier and Goud 1999, Nie *et al.* 2003). The Sar/Arf GTPase cycle is regulated by GEFs and GAPs (Jackson and Casanova 2000, Randazzo and Hirsch 2004).

Sar1 does not undergo any lipid modification, although it is associated with the endoplasmic reticulum to assist in the formation of COPII-coated vesicles from this organelle (Barlowe *et al.* 1994, Nishikawa and Nakano 1991). All Arfs are N-terminally myristoylated, a modification that is needed for membrane binding. Inactive Arf has low affinity to membranes because its lipid gets tucked into a hydrophobic pocket at

the protein surface. Activation of Arfs takes place only at membranes, and requires the lipid to be inserted into the membrane prior to GTP exchange. In this way activated Arfs are restricted to membrane surfaces (Roth 2000). All Arf proteins have nearly the same effector domain regions and can *in vitro*, to varying degrees, recruit coat proteins to Golgi membranes (Liang and Kornfeld 1997), activate phospholipase D (PLD) (Liang *et al*. 1997), and activate PIP5K (Honda *et al*. 1999). In the cell Arfs are targeted to distinct membranes where they function. The different GEFs and GAPs are also targeted to specific compartments where they presumably encounter specific Arf proteins (Donaldson and Honda 2005).

Mammalian Arfs can be subdivided into three classes based on their sequence similarity. They are thought to act through recruitment of soluble coat proteins to membranes facilitating vesicle formation, activation of lipid-modifying enzymes, and modulation of actin structures (Donaldson 2003).The class I Arfs (human Arf1, 2, and 3) seem to be functionally redundant, and are involved in COP I and some types of clathrin vesicle-coat assembly in the secretory and endocytic pathways (Schekman and Orci 1996, Rothman 1996). The biological role of class II Arfs (human Arf4 and 5) is still unclear. The class III Arf (human Arf6) influences membrane trafficking and the actin cytoskeleton at the plasma membrane (Donaldson 2003). There are no known coat proteins that are recruited by activated Arf6 to membranes, instead Arf6 is closely associated with modification of membrane lipid composition and actin cytoskeleton organization (Donaldson 2003).

Arf6 localizes to a tubular endosomal compartment in its GDP-bound conformation and with the plasma membrane in its GTP-bound conformation, regulating membrane traffic between these compartments through its GTPase cycle (D’Souza-Schorey *et al.* 1995, D’Souza-Schorey *et al.* 1998, Peters *et al.* 1995, Radhakrishna and Donaldson 1997). This membrane recycling pathway is used by many plasma membrane receptors including MHCI, interleukin-2 receptor, carboxypeptidase E and β1-integrin (Radhakrishna and Donaldson 1997, Brown *et al.* 2001, Blagoveshchenskaya *et al*. 2002, Arnaoutova *et al*. 2003, Powelka *et al.* 2004).

Arf6 also has an important role in remodeling of the cytoskeleton and cell motility downstream of Rac1 (Radhakrishna *et al.* 1999). A family of multi-domain proteins with Arf-GAP activity, are capable of interacting both with proteins involved in cell adhesion and actin reorganization (de Curtis 2001). At least some of these could function not only as Arf GAPs but also as Arf6 effectors in actin modulation (Hashimoto *et al*. 2004 a). Arf6 has also been implicated in the regulation of adherence junction disassembly/turnover, in epithelial cell migration (Palacios *et al*. 2001, Palacios *et al*. 2002) and in tight junction formation and stability downstream of E-cadherin (Luton *et al.* 2004). In addition, mutant forms of Arf6 that affect either actin or recycling inhibit motility of a breast cancer cell line (Powelka *et al.* 2004). Furthermore, tumor cell invasion has recently been shown to be regulated by Arf6 through activation of the ERK/MEK signaling pathway (Tague *et al*. 2004).

Arf6 localizes with PIP5K in cells and activates it, leading to production of $PIP_2$ (Honda *et al.* 1999). Arfs also bind to and activate phospholipase D (PLD) leading to production of phosphatic acid (PA) (Melendez *et al*. 2001, Powner *et al*. 2002, Xu *et al.* 2003). Because PA can activate PIP5K, Arf6 can, by regulating both PIP5K and

PLD, greatly amplify $PIP_2$ -mediated signals. A biophysical study (Ge *et al*. 2001) has suggested that Arf6 binding to $PIP_2$ vesicles alters bilayer structure providing an alternate way for Arf6 to affect membrane structure. Changes in membrane lipid composition and structure may mediate Arf6 alterations of the cortical actin cytoskeleton and regulation of membrane traffic and signal transduction (Donaldson 2003).

### *1.1.5 The Rab family*

The Rab family is the largest group of GTP-binding proteins in mammals. Based on ESTs and the sequenced human genome there are 63 members in humans (Zerial and McBride 2001). Rab proteins can be found in all eukaryotic cells and are major regulators of vesicle transport. It was in yeast that Rab proteins were first characterized as essential for secretion (Novick *et al.* 1980). Massive accumulation of vesicles in a

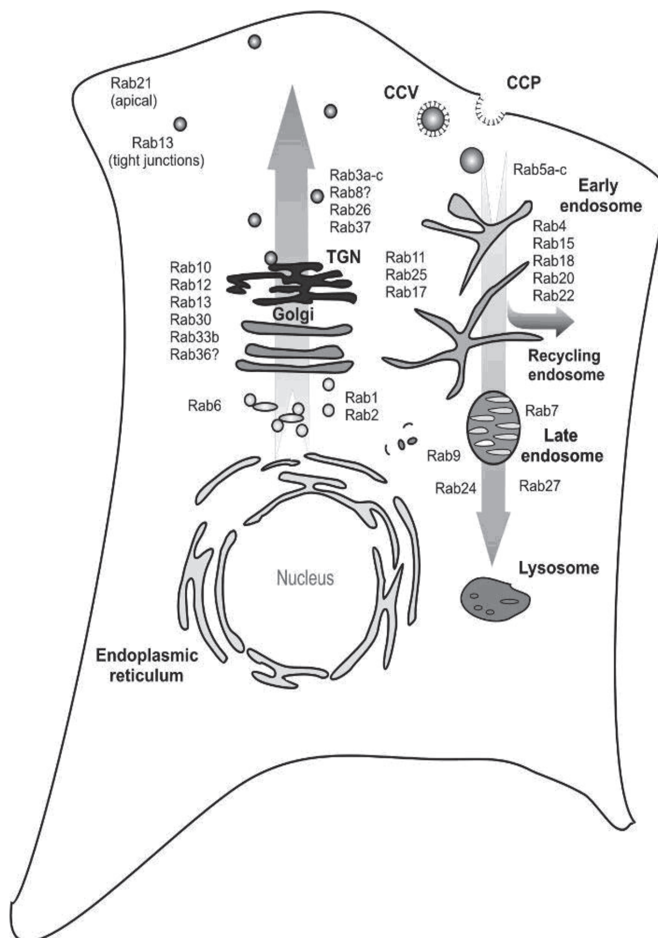



**Fig. 2. Rab trafficking in a mammalian cell.** This figure gives a summary of the intracellular localization of Rab proteins in mammalian cells (based on Zerial and McBride 2001). Some Rabs are cell specific (e.g. Rab3a in neurons), or tissue specific (e.g. Rab17 in epithelia). Others exhibit a cell type specific localization (e.g. Rab13 in tight junctions). CCV, clathrin-coated vesicle; CCP, clathrin-coated pit; TGN, trans-Golgi network.

distinct pathway was often seen as the result of a defective Rab protein (Lazar *et al.* 1997, Salminen and Novick 1987), and in some cases the Rab protein was also essential for cell survival. Recently evidence has started to emerge linking Rab dysfunction to human disease. Griscelli syndrome is a disease that is caused by mutations in Rab27a, affecting melanosomes and T cells. Mutations in general regulators of Rab activity such as REP-1, Rab GGTase, and Rab GDIα (leading to partial dysfunction of multiple Rab proteins) have also been linked to various disorders (e.g. retinal degeneration, mental retardation and Hermansky-Pudlak syndrome) (Seabra *et al.* 2002).

The Rab proteins function as molecular switches and timers by cycling between a GDP-bound and a GTP-bound conformation. Regulators of this cycle are GEFs, GAPs and GDI. In the cytosol the Rab protein is held as an inactive complex with GDI, it is released by an unknown mechanism, coupled to transport to its specific membrane compartment (Soldati *et al.* 1994, Ullrich *et al.* 1994). The GTPase is converted to the active form by the action of a GEF and the Rab is free to interact with its downstream effector(s). A GAP finally inactivates the Rab by hydrolyzing the bound GTP to GDP and inorganic phosphate. GDP Rabs can be extracted from the membrane by GDI, bringing it back to the cytosol, ready for another round of activation. Each Rab protein is believed to have a sub cellular localization corresponding to the step of vesicular transport it controls (see Fig. 2) (Novick and Zerial 1997, Nuoffer and Balch 1994, Olkkonen and Stenmark 1997, Takai *et al.* 2001). However the exact site of action for each Rab has not yet been defined. Moreover, it is almost entirely unknown how the Rab GTPases are targeted to their specific locations.

Rab GTPases are synthesized as soluble proteins in the cytosol and then post-translationally modified by prenylation. One or usually two geranyl-geranyl groups are added to cystein residues at the C-terminus of the Rab protein. Newly synthesized and prenylated Rabs are thought to be escorted by Rab escort protein (REP) to their target organelle where the lipid allows attachment into the membrane. Similarly GDI delivers Rab to the membrane for reuse in subsequent cycles of activation. Rab-GDI complexes are thought to be recognized by membrane linked proteins named GDFs (GDI displacement factors), a transient association allowing the Rab to become membrane associated. Once on the correct membrane the Rab would be activated by a GEF and interact with its effector and in this way achieve its proper localization. (Pfeffer 2005).

The specific mechanism by which the Rabs regulate vesicle transport is still unclear. They have been reported to control vesicle budding, vesicle and organelle movement along cytoskeletal tracks as well as their controlling vesicle docking and fusion (see reviews by Takai *et al.* 2001, Zerial and McBride 2001, Seabra and Coudrier 2004). In fact Rabs could have more than one function during a single round of membrane transport, undergoing several rounds of GTP-binding and hydrolysis. Microdomains on cellular membranes are believed to be created and maintained by Rabs and their effectors with the assistance of specific lipids and the cytoskeleton (Zerial and McBride 2001). These domains generate the correct environment for the particular vesicle transport step to take place. Interplay between such microdomains could then help regulate the organization of the whole cell, to balance the different transport events taking place at the same time. (Zerial and McBride 2001, Hall 2000).

**Table 1: Rab interacting proteins**

List of Rab-interacting proteins found so far in mammalian cells. Based on tables in review by Zerial and McBride, 2001.

| REGULATORS/ MODIFIERS | FUNCTION | RAB-SPECIFICITY | REFERENCES |
|---|---|---|---|
| Rab GGTaseII (geranyl-geranyl transferase) | Lipid modification of the Rab's c-terminus | All Rab proteins | Hall 2000 |
| REP-1 | Delivers newly synthesized, prenylated Rabs to membranes | All Rab proteins | Hall 2000 |
| RabGDI | Keeping the Rab in the GDP-bound form, recycling of the Rab | All Rab proteins | Hall 2000 |
| Mss4 | GDP-release factor | Rab1, Rab3, Rab8, Rab10 | Zerial and McBride 2001 |
| Rab3aGRF | Guanine nucleotide release factor | Rab3A | Burstein and Macara 1992 |
| Rabin3 | Unknown, possibly an exchange factor | Rab3? | Brondyk *et al*. 1995 |
| Rabex5 | GEF | Rab5,Rab4 | Zerial and McBride 2001 |
| RAP6 | GEF (also GAP for Ras) | Rab5 | Hunker *et al*. 2006 |
| AS160 | GAP | Rab2, Rab8, Rab10, Rab14 | Miinea *et al*. 2005 |
| Rab3-GAP | GAP | Rab3 | Burstein and Macara 1992 |
| Tuberous sclerosis 2 | GAP? | Rab5 | Zerial and McBride 2001 |
| RN-Tre | GAP (Also a Rab5 effector) | Rab5, Rab41 | Lanzetti *et al*. 2000, Haas *et al*. 2005 |
| RabGAP-5 | GAP | Rab5 | Haas *et al*. 2005 |
| GAPcenA | GAP | Rab6 | Zerial and McBride 2001 |

| EFFECTORS | FUNCTION | RAB SPECIFICITY | REFERENCES |
|---|---|---|---|
| PRA-1 and 2 | Proposed Rab receptors | Rab1, Rab3,Rab4, Rab5, Rab6, Rab7, Rab17, Rab22 | Bucci *et al*.1999, Abdul-Ghani *et al*. 2001 |
| p115 | Tethering, sequestering of SNAREs | Rab1 | Zerial and McBride 2001 |
| Rabphilin-3 | Potentiates fusion | (Rab3), Rab27 | Zerial and McBride 2001, Fukuda and Yamamoto 2005 |
| RIM 1 and 2 | Membrane fusion | Rab3 | Zerial and McBride 2001 |
| Calmodulin | Confers calcium sensitivity | Rab3 | Zerial and McBride 2001 |
| Noc2 | Inhibition of calcium-regulated exocytosis | (Rab3), Rab27 | Fukuda and Yamamoto 2005 |
| Rabaptin-4 | Protein sorting and recycling | Rab4 | Zerial and McBride 2001 |
| Syntaxin4 | | Rab4 | Li *et al*. 2001 |
| Dynein LIC-1 | | Rab4 | Bielli *et al*. 2001 |
| EEA1 | Tethering, core fusion component | Rab5 | Zerial and McBride 2001 |
| p150 | PI-3 kinase regulatory subunit | Rab5 | Zerial and McBride 2001 |
| p110β | PI-3 kinase catalytic subunit | Rab5 | Zerial and McBride 2001 |
| Rabaptin-5 and 5β | Recruits Rabex5, to activate Rab5 | Rab5, Rab4 | Zerial and McBride 2001 |
| Rabenosyn-5 | Required for CCV-EE and EE-EE fusion | Rab5, Rab4 | Zerial and McBride 2001 |
| RN-tre | Macropinocytosis, formation of circular ruffles, Also a Rab5-GAP | Rab5 | Lanzetti *et al*. 2004 |
| Rab6IP2 | unknown | Rab6 | Monier *et al*. 2002 |
| ERC family | unknown | Rab6 | Wang *et al*. 2002 |
| Rabkinesin-6 | Vesicle motility, cytokinesis | Rab6 | Zerial and McBride 2001 |

**Table 1 continuing**

| EFFECTORS | FUNCTION | RAB SPECIFICITY | REFERENCES |
|---|---|---|---|
| RILP | Transport to lysosomes | Rab7, Rab34 | Cantalupo *et al*. 2001, Wang and Hong 2002 |
| Rabring7 | lysosome biogenesis, traffic to late endosomes/lysosomes | Rab7 | Mizuno *et al*. 2003 |
| Rab8ip | Stress activated kinase | Rab8 | Ren *et al*. 1996 |
| Trip8b | Regulated secretion | Rab8b | Chen *et al*. 2001 |
| p40 | Stimulates fusion | Rab9 | Diaz *et al*. 1997 |
| Rab11BP/ Rabphilin-11 | Tfn recycling, membrane turnover | Rab11a and b, Rab25 | Zeng *et al*. 1999, Mammoto *et al*. 1999 |
| Rab11-FIP1 | Unknown | Rab11a and b, Rab25 | Hales *et al*. 2001 |
| Rab11-FIP2/nRip11 | Tfn recycling, coupling to myosinVb | Rab11a and b, Rab25 | Hales *et al*. 2001, Lindsay and McCaffrey 2002 |
| Rab11-FIP3/Eferin/ Arfophilin | Regulation of recycling endosome distribution, also bind Arf5 | Rab11, Rab25 | Prekeris *et al*. 2001, Hickson *et al*. 2003 |
| Rab11-FIP4 | Retinal development | Rab11 | Wallace *et al*. 2002, Muto *et al*. 2006 |
| Rip11/pp75 | Apical vesicle trafficking | Rab11a and b, Rab25 | Prekeris *et al*. 2000 |
| RCP (Rab-coupling protein) | Protein sorting in tubular endosomes | Rab11 (not Rab4) | Lindsay *et al*. 2002, Peden *et al*. 2004 |
| δ-PDE | Extracts Rab13 from membranes | Rab13 | Zerial and McBride 2001 |
| Melanophilin/Slac2-a | Recruits myosin-Va | Rab27a | Fukuda *et al*. 2002b |
| MyRIP | Trafficking of retinal melanosomes, coupling to myosin VIIa | Rab27a | El-Amraoui *et al*. 2002 |
| Rab33BP | Motility of vesicles | Rab33 | Zerial and McBride 2001 |

Three isoforms of Rab GDI have been isolated (Nishimura *et al.* 1994), several GAPs and even more effectors have been described (see Table 1, and review by Zerial and McBride 2001). Rab effectors are highly specialized molecules whose activities are adapted for the specific transport systems and organelles where they function. They are defined as a group by their specific binding to a GTP-Rab, and by their being required for a downstream function determined by that GTPase. Each Rab protein may interact with several effectors. Rab5-GTP has for example been shown to interact directly or indirectly with more than 20 polypeptides from bovine brain cytosol (Christoforidis *et al.* 1999). In Table 1 some of the known Rab-interacting proteins found to date are listed. However, many, if not most, are yet to be discovered and could offer important clues as to Rab protein function, which even to date remains unclear.

### *1.1.6 Crosstalk between small GTPases*

As more is known about individual GTPases, attention is now turning to how they are combined and regulated in a cooperative fashion. It is becoming increasingly clear that these molecules are not only linked in cascades but as a network, being coordinated and balanced, in order to create a functional and efficient entity.

An example of Ras-Rho crosstalk has been described in yeast, where the Ras-family member Rsr1 (activated by an unknown signal produced at the previous bud-site) interacts with Cdc24, a Cdc42 GEF, resulting in recruitment and activation of Cdc42 (Zheng *et al.* 1995). In yeast we can also find an example of Rho-Rab crosstalk.

Activated Cdc42 not only induces reorganization of actin but also recruits several small GTPases among these Sec4, a yeast Rab-protein, to the future bud site (Zheng *et al.* 1995). Another example of Rho-Rab crosstalk has been demonstrated in cultured MDCK cells, where Rho and Rab proteins have been shown to regulate cell adhesion and actin stress fiber formation in coordination (Imamura *et al.* 1998). It makes sense that Rab and Rho proteins would crosstalk to coordinate vesicle transport with actin cytoskeleton organization since actin rearrangement always precedes vesicle docking and fusion. A link between Ras signaling and vesicle transport is suggested by the fact that the catalytic domain of p120 RasGAP can specifically stimulate the GTPase activity on Rab5 (Liu and Li 1998). A hierarchy of activation has been reported with a cascade of the three Rho-family proteins Cdc42, Rac1 and RhoA. The order of activation corresponds to each protein's physiological role in motile cells. Activation of Cdc42 leads to formation of filopodia followed by Rac1 activation and the formation of lamellipodia and membrane ruffles and finally activation of RhoA needed for the formation of new adhesions at the protruding edge with the underlying matrix (Chant and Stowers 1995, Huttenlocher *et al.* 1995).

It is logical for different steps in vesicle transport to be balanced and coordinated in such a way that the organization of organelles, as well as the cell as a whole, remains functional and efficient. It seems obvious that Rab-family crosstalk should perform this function. Rabaptin-5, for example, interacts with both Rab5 and Rab4 in their GTP-bound conformations through two distinct regions (Vitale *et al.* 1998). Rab5 regulates clathrin-coated vesicle fusion with early endosomes as well as early endosome-early endosome fusion and Rab4 is involved in sorting and recycling in the early endosome; a balance of these functions is necessary for a functional endosomal compartment. In order to balance secretion and recycling of membrane receptors, there should be crosstalk between Rab3 regulating protein secretion and a Rab-protein regulating recycling from the plasma membrane. The Rab3 effector protein Rabphilin-3 binds Rabaptin-5 (Zerial and McBride 2001), which in turn recruits and binds Rabex-5. Together Rabaptin-5 and Rabex-5 activate Rab5, which provides one example of such crosstalk.

Several examples of crosstalk involving Arfs have also been reported. A large protein p619 links Arf1 and Rabs given that it can stimulate guanine nucleotide exchange on them (Rosa *et al*. 1996), and Arfophilin/Eferin/Rab11-FIP3 is a protein shown to bind Arf5 as well as Rab11 and Rab25 (Hickson *et al.* 2003). Arf-Rab crosstalk could possibly provide a way to coordinate the budding of vesicles with their transport in the cell. Two examples of crosstalk between Arf6 and Rac1 in the control of actin cytoskeleton reorganization are that the Arf GEF, EF6A induces cytoskeleton remodeling that can be blocked by expression of dominant negative Arf6 or Rac1 (Franco *et al.* 1999), and that dominant negative Arf6 inhibits growth factor- and Rac1-mediated membrane ruffling (Radhakrishna *et al.* 1999). Furthermore, there are several examples of crosstalk involving Arf6 and Rho or Ras GTPases in the regulation of cell migration and cell invasion. Santy and Casanova (2001) have shown that overexpression of ARNO, an Arf6 GEF, induces epithelial cell migration via activation of PLD and Rac1. Arf6 can furthermore, like the Ras GTPases activate the MEK/ERK-signaling pathway leading to enhanced invasive capacity (Tague *et al*. 2004), and the enhanced phospholipase activity in H-Ras transformed cells has been shown to be due to the synergistic activities of RalA and Arf6 (Xu *et al*. 2003).

## 1.2 The cytoskeleton

The cytoskeleton is composed of three different types of filaments; actin, microtubules and intermediate filaments. In each case these fibers are ordered polymers built from small protein subunits held together by non-covalent bonds. Together they stabilize the cell membrane and cell shape as well as to form tracks along which vesicles and organelles can move in the cytosol during intracellular trafficking. The cytoskeleton is constantly remodeled, and this is vital for a number of cellular processes including cell polarity, cell movement, cytokinesis, and tissue morphogenesis (Fuchs and Yang 1999).

### *1.2.1 Actin*

Actin cytoskeleton is composed of actin filaments and many actin-binding proteins. Filamentous actin (F-actin) is a polar structure built up from a single subunit; monomeric actin (G-actin). Actin is the most abundant protein in most eukaryotic cells and it has been very well conserved during evolution, in fact so well that actin molecules from different organisms are functionally interchangeable *in vitro* (Nefsky and Bretcher 1992). F-actin forms a tight $\alpha$-helix, a polar structure with each G-actin in the same direction. The plus end, also called barbed end, grows faster than the minus end, also called pointed end. ATP-actin is added onto the filament mainly at the plus end, within the filament the ATPs of the incorporated monomers are eventually hydrolyzed to ADP, and ADP-actin is finally disassembled at the minus end (Carlier 1998).

Assembly of actin into filaments occurs spontaneously under the right conditions. However, actin polymerization *in vivo* is a tightly regulated process with a myriad of different proteins taking part to ensure that the correct type of filament is formed or disassembled at the correct place at the right time. Actin cytoskeleton regulation occurs at multiple levels including the organization of actin into polymers and the organization of these polymers into bundles or networks. A large number of actin-binding proteins regulate actin assembly by controlling filament formation and cross-linking of the actin filaments (Welch *et al.* 1994, Schmidt and Hall 1998). In most cells actin filaments are concentrated mainly in a layer just beneath the plasma membrane, called the cell cortex. Here it is organized as a meshwork to stabilize the surface of the cell, influencing the shape and mechanical properties of the cell. Another main function for the actin cytoskeleton is transport of vesicles along actin filaments inside the cell (Kaksonen *et al*. 2006). In addition actin forms the contractile ring during cytokinesis, and powers cell motility (Burgess 2005, Ananthakrishnan and Ehrlicher 2007).

The motor-proteins, that move or slide along actin filaments in an ATP dependent manner (Kron *et al.* 1992), are called myosins. Myosins have been implicated in several processes like cytokinesis (Rodriguez and Paterson 1990), mitochondrial organization (Drubin *et al.* 1993, Simon *et al.* 1995, Smith *et al.* 1995), and vesicle trafficking (Johnston *et al.* 1991, Lillie and Brown 1994, Govindan *et al.* 1995).

Signaling through phosphoinositides and Ras-family small GTPases is crucial for actin cytoskeletal- and membrane- remodeling during cell motility (Qualmann and Kessels 2002). Rho family GTPases have been shown *in vivo* to regulate cytoskeleton remodeling during developmental and disease-related processes (Etienne- Manneville and Hall 2002). Rho, Rac, and Cdc42 signal through the actin network to regulate stress

fibers (Ridley and Hall 1992), lamellipodia (Ridley *et al.* 1992), and filopodia (Kozma *et al.* 1995, Brown *et al.* 2000) respectively. Recently it has been shown that Rho-family GTPases signal through the Arf family to bring about cytoskeletal rearrangements during cell motility (Zhang *et al.* 1999, Santy and Casanova 2001, Tarricone *et al.* 2001).

*1.2.2 Microtubules*

Microtubules are polymers of globular tubulin subunits arranged in cylindrical tubes. They are, like actin-filaments, polar structures whose ends have different rates of assembly. Like actin, tubulin binds a nucleotide, in this case GTP, which is hydrolyzed when the subunit has been incorporated into the filament. The building blocks for microtubule assembly are dimers formed from α-tubulin and β-tubulin. These αβ-tubulin dimers form protofilaments that are added on to γ-tubulin rings to form the microtubule wall. More dimers can then be added to elongate the microtubule. The γ-tubulin rings are found at the microtubule organizing center, also called the centrosome, near the center of the cell. The αβ-dimers are added on in a specific orientation creating an asymmetry where the minus end normally is embedded in the centrosome and both growth and shrinkage occur preferentially at the plus end. The microtubule organizing center is the major organizing structure of the cell, determining the organization of microtubule-associated structures and organelles like mitochondria, the Golgi complex, and the endoplasmic reticulum (Lodish *et al.* 1999). Microtubules are continuously assembled and disassembled in a process called dynamic instability. As a consequence, the microtubule organizing centre, or centrosome, is continually shooting out new microtubules in an exploratory fashion in different directions and retracting them. Attachment to another molecule or cell structure can prevent microtubule disassembly.

The motor proteins of the microtubule system can be divided into two different groups; kinesins and dyneins. Kinesins are dimers that show specificity for their respective cargo. They are nearly all plus end directed (moving toward the centrosome, inward) whereas dyneins are minus end directed (moving away from the centrosome, outward). Dyneins are large multimeric proteins that require large complexes of microtubule-binding proteins for movement. Both types of motor use energy derived from repeated cycles of ATP hydrolysis to move. Microtubules are used as tracks for membrane transport bi-directionally, but they are not absolutely required for short-range transport (Cole and Lippincott-Schwartz 1995, Bloom and Goldstein 1998). Microtubules are, however, needed for long-range transport; a striking example being neuronal transport where supplies have to be delivered very long distances from the cell body out to the neurite. New evidence implicates dysfunction of microtubule-dependent transport in the development, or even the cause, of several neurodegenerative diseases (Guzik and Goldstein 2004).

The polarity of the cell is linked to the orientation of the microtubules and in a motile cell the microtubules are oriented towards the leading lamellae in the direction of movement (Gundersen and Bulinski 1988). New evidence is emerging to indicate Rho family GTPases as central players in the regulation of microtubule dynamics (Gundersen *et al.* 2004, Kodama *et al.* 2004). Since it is well established that Rho proteins are central in control of actin cytoskeleton rearrangements, it seems likely that

Rho-family GTPases could be key molecules in the coordination of actin-microtubule crosstalk. Coordination between microtubules and filamentous actin can be seen in many polarized processes including cell shape, motility, growth-cone guidance and wound healing (Kodama *et al.* 2004).

### *1.2.3 Intermediate filaments*

Intermediate filament subunits are alpha-helical rods that assemble into ropelike filaments, resembling microtubules in structure. Intermediate filaments are extremely stable, their principal function is structural; to reinforce cells and to organize cells into tissues. Therefore they are foremost found in the cytoplasm of cells that need to withstand mechanical stress e.g. muscle cells, and epithelial cells of the skin, as well as along the length of nerve cell axons. The nuclear lamina is a mesh of intermediate filaments that underlies and strengthens the nuclear envelope in all eukaryotic cells. Intermediate filaments can be classified into six types based on sequence similarity: type I (acid keratins), type II (basic keratins), type III (vimentin, desmin, glial fibrillary acid protein, peripherin), type IV (NF-L, NF-M, NF-H, internexin), nonstandard type IV (filensin, phakinin) and type V (laminA, B and C). These classes vary to a great extent in sequence and molecular weight. The expression of intermediate filaments is characteristic of a certain tissue or cell type, therefore they can sometimes be used to identify the cellular origin of tumors. (See Lodish *et al*. 1999).

## **1.3 Vesicle transport**

For any cell in our body it is essential that its proteins are targeted and sorted to the correct membrane or aqueous compartment. This is achieved by vesicular transport. Newly synthesized proteins enter the endoplasmic reticulum (ER) where they are sorted and transported to their correct intra- or extra-cellular destination (secretory pathway). Material can also be taken up from outside the plasma membrane and transported into the cell (endocytic pathway). Finally, there are recycling pathways for proteins that have escaped their proper localization or have served a function elsewhere in the cell, to be transported back to their appropriate locations for reuse.

Vesicular transport progresses in several different phases. First, a vesicle buds off the donor compartment. It is then transported along cytoskeletal tracks to its proper destination where it docks and finally fuses (Rothman 1996). Budding is a process regulated by the Sar1/Arf family of GTPases. They recruit coat components to the membrane leading to the formation of a specific domain on the membrane that is enriched in certain membrane receptors and their associated proteins. A bud is formed and pinched off, to form a vesicle that can then be transported through the cytosol, along cytoskeletal tracks. The dynamin-family of large GTPases (reviewed by Praefcke and McMahon 2004) has been proposed to constitute universal scission molecules. Once the vesicle has been formed it is uncoated, an essential step for the vesicle to be able to dock with its acceptor compartment. Once the vesicle reaches its destination, there is conserved machinery in place that mediates membrane fusion. Central to this are the SNAREs and SNAP25. SNARE-homologues make up a family of proteins that in mammals so far consists of more than 30 members (Bock and Scheller 1997) localized

to different intracellular compartments. A vesicle SNARE (v-SNARE) forms a trimeric complex with a target-membrane SNARE (t-SNARE) and SNAP25, bringing the two membranes into close proximity, facilitating their fusion (Weber *et al.* 1998). Thereafter, NSF and SNAPs allow the SNARE/SNAP-complex to dissociate and the components to be recycled (Nichols *et al.* 1997).

The localization pattern of SNAREs may contribute to vesicle docking specificity, but since SNARE protein interactions are not selective, it is obvious that other factors are also needed to confer the additional specificity (Yang *et al.* 1999). Tethering factors such as Uso1p, TRAPP, p115, exocyst and EEA1 all bind membranes before the formation of the SNARE complex. EEA1 is an effector of Rab5 (Christoforidis *et al*. 1999), and Uso1p is the yeast homologue of p115, that has been shown to bind directly to Rab1 (Allan *et al*. 2000). Consequently, the Rab family of small GTPases is likely to aid vesicle targeting through these tethering proteins. Evidence is also accumulating linking Rab proteins to cytoskeleton motor proteins, implicating them in the transport of vesicles through the cytoplasm along cytoskeletal tracks. Rabkinesin6, a Rab6 specific effector protein, for example is a kinesin-like protein that functions as a motor protein in transport of tubular structures from the Golgi apparatus to the cell periphery (White *et al*. 1999). Rab11 and Rab25 have been shown to interact with the C-terminal domain of myosinVb (Lappiere *et al*. 2001), and Rab27a functions as an essential component of the melanosome receptor for myosinVa, interacting indirectly via one or more bridging proteins (Wu *et al*. 2002). As more and more information becomes available it seems increasingly likely that Rabs are multifunctional molecules involved in many of the steps involved in vesicle formation, transport and fusion at the target membrane. They could be activated in several rounds during one membrane transport step and thus be involved in more than just one step in this process through the interaction of different effector proteins.

### 1.4 Cell adhesion and ECM proteins

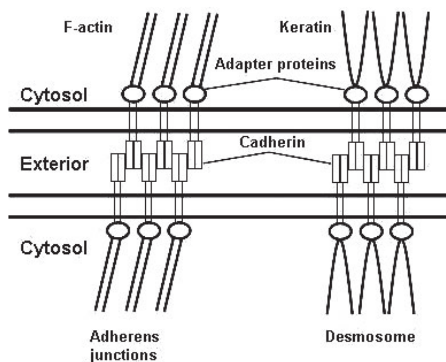
Cells adhere to the surrounding matrix and neighboring cells by adhesion receptors on the cell surface. There are five principal classes of adhesion receptors: integrins, cadherins, the immunoglobulin superfamily, selectins, and proteoglycans (see review by Gumbiner 1996). The ECM proteins, to which the receptors bind, are usually large glycoproteins such as collagens, fibronectins, laminins and proteoglycans. Cytoplasmic peripheral membrane proteins link the adhesion systems to the cytoskeleton. There are four main types of junctions: the tight junction (TJ), the gap junction, cell-cell junctions (adherence junctions and desmosomes), and cell-matrix junctions (focal adhesions and hemidesmosomes). Tight junctions connect epithelial cells preventing the passage of fluids through the cell layer. Gap junctions allow for direct communication between cells in tissues, by permitting adjacent cells to exchange small molecules. Cell-cell and cell-matrix adhesion perform a structural role of holding cells within a tissue. Cadherins and integrins are the major molecules that connect the cell exterior with the internal cytoskeleton. (Lodish *et al.* 1999).

Cadherins make up a large family of adhesion receptors, with over 80 members known (Angst *et al*. 2000). They mediate calcium-dependent cell-cell adhesion, known to be vital both during development (Takeichi 1991) and for the adult organism

(Gumbiner 1996). The three main cadherins on mammalian cells are E-cadherin, P-cadherin and N-cadherin. E-cadherin is predominantly expressed in non-neuronal epithelial tissue. It is required for epithelial cells to remain tightly associated in the epithelium, and is believed to act as a suppressor of tumor cell invasiveness and metastasis (Birchmeier and Behrens 1994, Takeichi 1993). Calcium ions cause E-cadherin monomers to form parallel homodimers, cell adhesion then results from head to head contact between E-cadherin dimers in adjacent cell membranes (Shapiro *et al.* 1995).

In adherence junctions (AJs) cadherins are liked to actin/myosin filaments via the adapter molecules, α- and β-catenin (Gumbiner 1993, Kemler *et al.* 1989). Nectins are a family of Ca2+-independent Ig-like cell adhesion proteins, with four members, that are also involved in cell-cell adhesion. They are linked to the actin cytoskeleton via afadin adaptor proteins and organize AJs cooperatively with the cadherin-catenin system in epithelial cells (Miyoshi and Takai 2005). The desmosome is a cadherin-containing junction, where cadherin is linked to the intermediate filament network (usually keratin) via catenin-like adapter molecules. This network gives the extra strength and rigidity needed to survive high levels of mechanical stress, for example, in epithelium and cardiac muscle (Gumbiner 1996). Tight junctions (TJs) are thought to be membrane microdomains, a dynamic assembly of cholesterol and sphingolipids. Three types of transmembrane proteins have been identified at TJs: occludin, claudins and junctional adhesion molecules. They are all linked to the actin cytoskeleton via adaptor ZO proteins. Apart from their obvious role as barriers, TJs also have a role in concentrating signaling and cell polarity proteins, and help coordinate many cell processes (Miyoshi and Takai 2005).

A. cell-cell adhesion junctions




B. cell-matrix adhesion junctions

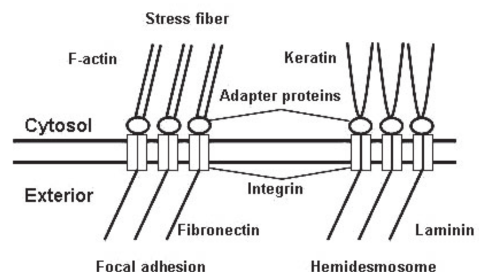



**Fig. 3. Schematic representation of junctions in cell adhesion.** A. The two main types of cell-cell adhesion junctions; adherence junctions and desmosomes. Cadherins are linked to actin or intermediate filaments (usually keratin) respectively via adapter proteins. Two other junction types linking cells in tissues are gap junctions and tight junctions, not depicted here. B. The two main types of cell-matrix adhesion junctions; focal adhesions and hemidesmosomes. Here integrins link actin and intermediate filaments respectively to the extracellular matrix via adapter proteins. (Based on figures in Lodish *et al.* 1999)

Integrins are the main cell adhesion molecules responsible for cell-matrix interactions. In animals there are at least 22 heterodimers composed of 17 types of α- and 8 types of β-subunits. This diversity enables the integrins to specifically bind a multitude of ligands. Most integrins are expressed on a variety of cells and most cells express several integrins, enabling them to bind to several matrix surfaces (Hynes 1992, Hynes 2002). Integrins normally show relatively low affinity for their ligands and need to be 'activated' to bind the ECM. Activation occurs either by a conformational change associated with increased binding affinity or with an increase in the number of integrins at the site of adhesion, a process called clustering (Lodish *et al.* 1999).

Integrin-containing junctions connect cells to the substratum. There are two types of such junctions: hemidesmosomes, mainly found in epithelial cells, (intermediate filaments connected to the basal laminae) and focal adhesions (actin cytoskeleton attached to fibers of fibronectin) (Lodish *et al.* 1999). Ligand binding induces integrin clustering and recruitment of actin filaments and signaling proteins to the cytoplasmic domain of the integrins (Hynes 2002). These ECM attachment sites are known as focal complexes when they are still forming and then as focal adhesions when they have matured into larger complexes. The integrin-actin connection is very dynamic and highly regulated. It is even differently regulated at different locations in the cell. At the leading edge of a migrating cell for example, integrin binding to the ECM leads to local rearrangement of the actin cytoskeleton, promoting protrusion formation, whereas at the rear integrins detach from the ECM, dissolve the link to the cytoskeleton and are at least partially recycled to the front (Ballestrem *et al.* 2001, Laukaitis *et al*. 2001).

Integrins are signaling molecules that can transmit signals both from the outside inwards and from inside the cell to the ECM (Clark and Brugge 1995). Adaptor proteins like ILK, talin, α-actinin and filamin link the integrin receptors to the actin cytoskeleton and signaling molecules, creating platforms for integrin signaling (Brakebusch and Fässler 2003). Focal adhesions can thus be seen as multiprotein platforms regulating cell-adhesion dependent signals for cell growth and motility.

Some common integrin ligands are collagens, laminins, and fibronectins. Collagens constitute the majority of insoluble protein in the extracellular matrix. Most collagen in the body belongs to a type of collagen that forms fibrils. After being secreted into the extracellular space, these collagen molecules pack together to form long thin fibrils, that are then organized into networks. Another type of collagen assists in organization of fibrils by binding to and cross-linking fibrous collagen. Finally there is a type of collagen that forms two-dimensional networks. It is this type of collagen that together with the laminin family forms the two dimensional lattice of the basal lamina. This structure is a thin sheet-like network of ECM components, which is important for organizing cells into tissues and for guiding migrating cells during development as well as for many more specialized functions. Laminin is a cross-shaped, large multi-adhesive matrix protein, predominantly found in the basal lamina. (Lodish *et al.* 1999).

Fibronectins are soluble multi-adhesive matrix proteins, whose primary role is to attach cells to matrices containing fibrous collagen. They facilitate migration and cellular differentiation of many cell types during development. In the adult organism they are important for wound healing because they facilitate migration of macrophages and other immune cells into the affected area. To conclude, there is the proteoglycan

family of extracellular matrix proteins. They are large highly hydrated molecules found in the ECM and on the surface of many cells. The most common type of proteoglycan on the cell surface is the syndecan family. Syndecans can bind both collagen and fibronectin and also interact with the actin cytoskeleton; thus, they can facilitate cell-matrix interactions. (Lodish *et al.* 1999).

## 1.5 Cell polarization

Cell polarity is a process in which a cell organizes its components asymmetrically into functionally specialized domains. It all starts with an external cue leading to the recruitment of certain components to a marked place on the cell surface. This place then recruits further signaling components and also reorganizes the cytoskeleton. At this point membrane traffic is redirected in such a way that the molecular structure now in place is maintained and reinforced. The best-studied polar cell types are budding yeast, epithelial cells, and neurons, but polarity is something that is needed for most cells at some point. Polarity is for example essential for cell division, differentiation and migration. (Nabi 1999).

### *1.5.1 Polarization of budding yeast*

Yeast is a useful model organism to study asymmetry and polarization since many of the components and processes important for polarized growth in yeast have been found to be conserved in other eukaryotes (Horton and Ehlers 2003). During both budding and mating the yeast *S. cerevisiae* goes through polarized growth (for reviews see Madden and Snyder 1998, Chant 1999). The first event in this process is establishment of a site for growth on the cell surface. Next the cytoskeleton is reorganized towards this site and finally membrane transport is redirected towards the growth site. The actin cytoskeleton is central to this process, it is redistributed very early, directing secretion to the bud and making up a docking site to help establish the asymmetry and then maintain it. Cdc42 is an important component always found at the tip of the protruding membrane. Cdc42 localizes in an actin-independent manner (Ayscough *et al.* 1997) and has been suggested to define the alignment of cytoskeletal polarization. Cdc42 was in fact one of the first genes discovered as critical for polarity establishment, as Cdc42 mutant yeast fail to develop a bud site, and instead become large, spherical and multinucleate (Adams *et al.* 1990).

Together with the Rab protein, Sec4p, a complex of proteins, the exocyst, is responsible for the docking of secretory vesicles at the bud site (Terbush *et al.* 1996, Guo *et al.* 1999). A component of the exocyst, Sec3p, is tightly localized to sites of the plasma membrane where the vesicles fuse (Finger *et al.* 1998). Sec3 is potentially the membrane-docking factor for vesicles. The microtubule cytoskeleton is also an important component in polarized growth. It is known that microtubules are stabilized at the mother-bud contact site or at the tip of the mating protrusion. Myosin motors, moving along actin filaments, direct at least some types of secretory vesicles to the budding or mating site (Govindan *et al.* 1995, Finger and Novick 1998, Pruyne *et al.* 1998).

### *1.5.2 Polarization of mammalian cells*

One of the most extensively studied polarized mammalian cell types is the epithelial cell. Polarized epithelial cells are joined together side to side to form multi-cellular sheets. These sheets cover the entire external surface of the body as well as lining the internal cavities and perform many important functions for the organism, including forming a protective barrier against invading pathogens, secreting specialized protein products like hormones and milk, and absorbing nutrients from the gut. Essential for the function of the epithelial sheet, is the fact that it has two faces, apical and basal, which are chemically different due to a polarized internal organization of the individual epithelial cells. (Miyoshi and Takai 2005).

Four types of junctions can be identified in electron micrographs in epithelial cells: tight junctions (TJs), adherence junctions (AJs), desmosomes, and gap junctions. TJs provide barriers preventing leakage of molecules across the epithelium as well as a barrier between the apical and basolateral domains of the plasma membrane. AJs form continuous adhesion belts just below the TJs, they have a role in stabilizing TJs and additionally serve as a regulation centers since they are associated with many proteins involved in actin organization and signal transduction (Miyoshi and Takai 2005). Desmosomes provide extra strength and rigidity needed to survive high levels of stress for example in epithelium whereas gap junctions allow direct communication between cells in tissues (Lodish *et al.* 1999).

Polarization of an epithelial cell is started by spatial cues induced by cell-cell or cell-matrix contacts. These cues stimulate localized assembly of the cytoskeleton as well as a targeting patch for transport vesicles. The microtubules and secretory apparatus reorganize in the cytoplasm relative to the cues, and the accurate sorting and delivery of protein to their correct compartments reinforce and stabilize the asymmetry of the cell surface (Yeaman *et al.* 1999). The actin cytoskeleton serves several important functions in the establishment of epithelial cell polarity. It strengthens the adhesive contacts, acts as a scaffold or targeting patch for the recruitment and binding of signaling proteins that further define the different membrane domains, and promotes the assembly of structures that physically restricts intermixing of newly synthesized apical and basolateral membrane proteins (Yeaman *et al.* 1999). Rho proteins are key regulators of the actin cytoskeleton and are also important for establishing and maintaining cell polarity (Etienne-Manneville and Hall 2002, Nobes and Hall 1999, Jou and Nelson 1998).

A protein complex (Par3/Par6/aPKC) implicated in cytoskeleton regulation (Qiu *et al*. 2000), has been proposed as an evolutionary conserved polarization signal (Wodarz 2002). The Par3/Par6/aPKC-complex is present in the cytosol of epithelial cells and recruited to cell-cell contacts during the formation of epithelial tight junctions (Yamanaka *et al.* 2001). In *C. elegans* Par regulation of microtubule organization is crucial for polarized cell division (O'Connell *et al*. 2000), and in migrating astrocytes the proper orientation of microtubules by the Par complex may direct membrane addition to the leading edge of the cell (Schmoranzer and Simon 2003, Horton and Ehlers 2003). Furthermore Cdc42 may, in addition to its role in actin rearrangements, also act through the Par complex to regulate microtubule organization (Etienne-Manneville and Hall 2003).

Besides the Par complex, two additional multiprotein-complexes have been identified as important for establishment of polarity in *Drosophila:* the Crumbs complex (Knust *et al*. 1993) and the Scribble complex (Bilder *et al*. 2000, Bilder and Perrimon 2000). Mammalian homologues of these complexes have also been found. Crumbs and Scrib have furthermore been shown to play opposing and balancing roles in the establishment of apical and lateral membrane identity, respectively (Bilder *et al*. 2000). In epithelial cells these protein complexes are located in close apposition but on opposite sides of the tight junction (Lee *et al*. 2002, Medina *et al*. 2002).

During cell polarization, microtubules reorganize (Bacallalo *et al*. 1989) simultaneous with reorganization of the secretory apparatus. Components of the secretory apparatus, like the Golgi apparatus and endosomes, become restricted to different regions in the cytoplasm (Davies and Garrod 1997). In fact the microtubule cytoskeleton is important for determining Golgi distribution (Shorter and Warren 2002) and vesicles from the TGN traffic along microtubules to the plasma membrane (Schmoranzer and Simon 2003), indicating that microtubules are very important in creating a polarized secretory pathway oriented to facilitate directional membrane traffic. Rab family GTPases are also likely to play a key role in controlling membrane traffic, which is set up to stabilize and maintain the asymmetry of the plasma membrane. The exocyst proteins, that in yeast define a site for docking of secretory vesicles on the cell surface, are highly conserved from yeast to mammals, and are likely to define membrane fusion sites at the plasma membrane also in mammalian cells (Terbush *et al.* 1996, Hsu *et al*. 1996).

In general, protein sorting occurs along the secretory pathway, the endocytic pathway or a combination of both. Newly synthesized proteins are sorted in the Golgi complex and at the TGN. It is at the TGN that segregation of apical and basolateral proteins occur in most epithelial cells (Keller *et al*. 2001). However in a number of cell types trafficking along the endocytic pathway is critical for proper localization of membrane proteins. In these cases membrane proteins are inserted randomly in the plasma membrane, rapidly internalized in a clathrin-dependent manner and trafficked via transcytosis to the proper membrane domain (Tuma and Hubbard 2003). Extensive sorting can take place along the endosomal system, as not all endosomes are created equal (Rojas and Apodaca 2002, Horton and Ehlers 2003).

Targeted delivery involves protein sorting, targeting of the vesicle to the correct domain and docking/fusion with the correct membrane domain. Very little is known about the molecular mechanism of polarized vesicle transport in epithelial cells. V-and t-SNAREs define a set of membranes that have the potential to fuse, but other proteins are required to define the site at which fusion occurs. Munc-18 proteins could regulate vesicle fusion by controlling the ability of syntaxins to interact with other components of the SNARE complex and Rab proteins are likely regulators of the timing and or localization of vesicle fusion. (Yeaman *et al.* 1999, Horton and Ehlers 2003).

Different sorting signals for apical and basolateral membrane proteins have been identified, but they are only partially known and the mechanisms for sorting are still unclear. Apical trafficking has been suggested to be taken care of by lipid rafts, glycolipid and cholesterol rich membrane domains involving cavelolin, whereas basolateral protein traffic is supposedly handled by classical vesicle budding from the

ER going through the Golgi complex and onto the plasma membrane (Yeaman *et al.* 1999, Horton and Ehlers 2003). A form of membrane transport only taking place in polarized epithelial cells is transcytosis (Mostov 1995), a process of transport between the apical and basolateral membranes.

Microtubules have been shown to be involved in basolateral to apical transcytosis and apical recycling, but their role in TGN to plasma membrane delivery is controversial (Breitfeld *et al.* 1990, Matter *et al.* 1990). Microtubules seem to facilitate, but not specify the delivery of vesicles to either the apical or basolateral membrane. Finally there has to be a system in place restricting diffusion of lipids and membrane proteins within the plasma membrane, once the proper organization of the polarized cell is in place. In polarized epithelial cells the tight junction (TJ) is such a barrier.

Neurons are another example of a polarized mammalian cell type, where the axon and the dendrites make up the apical and basolateral surfaces of epithelial cells respectively. Axon specification, or the establishment of a single axon, is the first step toward a polarized neuronal cell (Fukata *et al*. 2002). In the axon, microtubules are oriented with the plus end always facing the tip, whereas microtubule orientation is less ordered in the dendrites (Baas *et al.* 1989). The stabilization of microtubules in the growth cone facilitated by the actin cytoskeleton has been suggested to be a possible mechanism of axon specification (Bradke and Dotti 1999).

Polarized vesicular transport is believed to be responsible for maintaining the polarization for the lifetime of the neuron. A mechanism to achieve this polarized traffic has been suggested (Horton and Ehlers 2003); that specific SNARE proteins be either differentially distributed, or functionally different in axon and dendritic domains. No specific fusion barrier such as the tight junction in epithelial cells has yet been observed in neurons. However, a barrier restricting diffusion of lipids has been seen in mature neurons (Nakada *et al*. 2003). Another example of a polarized mammalian cell is the motile cell, where membrane surfaces have to be remodeled continuously to facilitate the migration of the cell.

### *1.5.3 The motile cell*

Cell migration is a multistep process that is essential during embryonic development as well as for important processes such as skin renewal, tissue repair and the immune response in the adult organism. It is also an important contributing component in several pathological processes, the most obvious example being cancer. In animal cells cell migration is directed by extracellular cues functioning either as attractants or repellants. These can be soluble factors working at a distance or local signals from neighboring cells or the ECM. The two major chemoattractants for eukaryotic cells are chemokines, acting through seven-membrane G protein receptors (Ward and Westwick 1998), and peptide growth-factors and cytokines acting through tyrosine-kinase receptors (Rosen and Goldberg 1989). Migration of a cell toward a source of chemoattractant is also called chemotaxis.

Cell migration can be regarded as a cyclical process (Lauffenburger and Horowitz 1996). To be able to move, the cell first has to polarize; to form a leading edge and a trailing tail (see Nabi 1999, Ridley *et al*. 2003 for reviews). At the front, a region called

leading edge or pseudopod is formed by the reorganization of the actin cytoskeleton into filopodia and lamellipodia. Next, the membrane makes new contacts to the underlying matrix. Signaling and adhesion receptors are reorganized to the front of the cell. At the rear of the cell, adhesions, in contrast, have to be broken for the cell to be able to move on. Tractional force is accomplished by myosins interacting with actin filaments attached to sites of adhesion on the substratum (Lauffenburger and Horowitz 1996, Ridley *et al*. 2003). There has to be a constant turnover of focal adhesions and stress fibers or these structures will be inhibitory to cell migration. This activity is promoted by the Ras small GTPase (Nobes and Hall 1999).

Rho GTPases have emerged as central regulators of many aspects of cell migration. The best characterized function is their regulation of actin dynamics. RhoA regulates the assembly of contractile actin-myosin filaments, whereas Rac1 and Cdc42 regulate the polymerization of actin to form peripheral protrusions, lamellipodia and filopodia respectively. All three promote the assembly of integrin-based adhesion complexes. (Nobes and Hall 1995). Cdc42 activity is furthermore required for the establishment of cell polarity and all three affect the microtubule cytoskeleton and gene transcription (Etienne-Manneville and Hall 2002). In microtubule cytoskeleton reorganization RhoA and Rac1 promote microtubule stabilization and elongation whereas Cdc42 regulates microtubule and centrosome polarity (see review by Raftopoulou and Hall 2004).

Actin binding proteins have a very important role in the control of actin-polymerization at the leading edge, making sure that the correct structures are made and disassembled respectively at the correct time and place in the cell to facilitate the formation of the motile structures, filopodia, lamellipodia and membrane ruffles. Actin filaments have to be polymerized at the leading edge in a regulated fashion, oriented so that the fast-growing end is pointed towards the protruding front (Small *et al*. 1978). The actin network changes during its retrograde flow and develops stronger adhesion sites to the underlying matrix (focal adhesions) (Symons and Mitchinson 1991, Chan *et al*. 2000). Cellular motility is thus believed to be driven by assembly and disassembly of

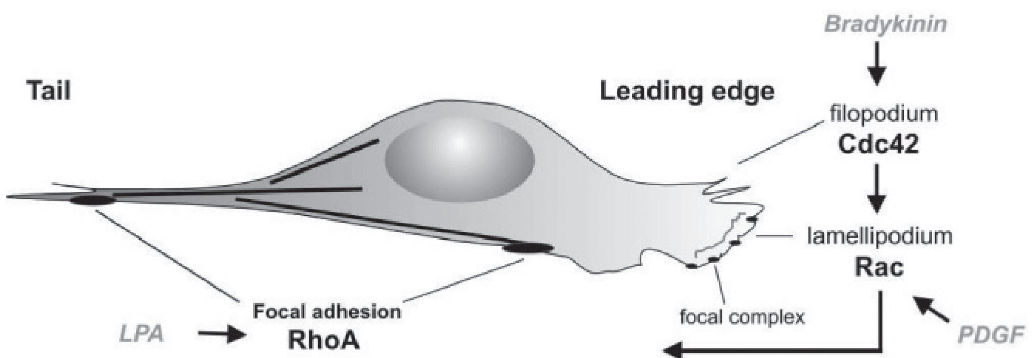



**Fig. 4. A motile cell.** The Rho-family of small GTPases controls the formation of distinct but interdependent actin-containing structures crucial for the motile cell. Cdc42 controls the formation of filopodia, rapidly followed by the formation of lamellipodia, controlled by Rac. Finally RhoA controls the formation of focal adhesions and stress fibers. These activities have to be strictly controlled and coordinated to lead to the motile cell phenotype and the forward translocation of the cell.

actin filaments (Pollard and Borisy 2003). Rapid growth of actin has furthermore been suggested to be the force responsible for pushing the membrane forward (Cunningham *et al.* 1992).

Recently it has also been shown that polarization of cholesterol-enriched domains has an important role at the leading edge to provide a suitable viscoelasticity of the plasma membrane for deformation by actin (Vasanji *et al*. 2004). Such cholesterol-enriched domains could thus perform dual functions as signaling platforms as well as microdomains providing the optimal physiological properties at the site for protrusion. Douglass and Vale (2005) have followed GFP-tagged signaling proteins in Jurkat T cells by single-molecule and scanning confocal imaging. They found evidence of microdomains that are created by protein-protein interactions. F-actin seems to be needed for the formation of these microdomains, but their maintenance is not dependent on either actin or lipid rafts. They could see that the microdomains had the capacity to limit free diffusion of molecules in the membrane by trapping or excluding certain proteins, thereby facilitating T-cell signaling.

The role of microtubules in cell migration is far from clear. It is known that the microtubule cytoskeleton is polarized in a migrating cell. The centrosome is oriented in the direction of movement in some though not all cell types (Euteneuer and Schliwa 1992), and the microtubules themselves are preferentially organized so that their stabilized plus ends are facing the leading edge (Gundersen and Bulinski 1988). Three hypotheses have been put forward for the function of polarized microtubules in migrating cells. One suggestion is that the microtubules provide tracks for directed transport of membranes and organelles to the leading edge (Nabi 1999). This though, is unlikely to be the sole function of polarized microtubules (Liao *et al*. 1995). A second hypothesis is that the polarized microtubules directly promote lamellipodial protrusion, which is required for both stabilization of the leading edge and maintaining polarized movement of the cell (Rinnerthaler *et al*. 1988). A further possibility is that microtubules are responsible for the local regulation of retraction and adhesion (Bershadsky *et al*. 1996).

### 1.6 Cell migration and membrane traffic

The role of membrane traffic in cell migration has yet to be thoroughly investigated. It is known, however, that newly synthesized vesicular stomatitis virus G (VSVG) proteins are delivered to the leading lamella of migrating cells, whereas influenza membrane proteins are delivered to the trailing edge (Bergman *et al.* 1983, Millan *et al*. 2001). The positioning of Golgi towards the leading lamella has also been considered an indication of directional membrane transport during cell motility. There is evidence that some endocytosed plasma membrane receptors are recycled to the leading lamella (Hopkins *et al.* 1994) and it has also been shown that at least some integrins are recycled from the trailing edge towards the leading edge via a specific recycling compartment (Bretscher *et al.* 1998, Pierini *et al*. 2000). This kind of integrin recycling could be important both for cell detachment and the creation of new attachment sites at specific places of the plasma membrane. Finally, a rapid flow of bulk membranes through a recycling system could be important for the creation of new cell surface domains, like cell protrusions and extensions during polarized cell migration (Thompson and Bretscher 2002). Brefeldin A

(BFA), a drug which inhibits both exocytosis and some forms of recycling also inhibits cell polarity and migration. A temperature sensitive mutant of NEM-sensitive factor (NSF) has been shown to inhibit the formation of polarity and decrease cell locomotion in *Dictyostelium discoideum,* demonstrating that membrane recycling plays at least some role in the generation of cell migration (Thompson and Bretscher 2002).

Although the original hypothesis that membrane flow would be the only driving force of cell migration has been rejected, results are emerging that connect actin and microtubular dynamics to membrane traffic in the control of cell shape changes (Nabi 1999). Firstly, Rho family proteins have been demonstrated not only to regulate the cytoskeleton but also different membrane transport routes in epithelial cells (Kamei *et al.* 1999, Kroschewski *et al.* 1999). Secondly, the Rab GTPase Rab8 promotes polarized transport of membrane proteins through reorganization of actin and microtubules (Peränen *et al.* 1996). Finally, Arf6 controls a novel membrane-recycling pathway mediating actin dynamics (Radhakrishna *et al*. 1999).

Best studied so far has been the Arf6-pathway. Expression of certain activated Arf6 mutants leads to actin reorganization and enhanced cell motility (Santy and Casanova, 2001). This is dependent on the activity of Rho proteins, especially Rac1 and RhoA (Boshans *et al.* 2000). Arf6 has also been shown to cross-talk with the small GTPases H-Ras and RalA in the control of phospholipase D activity (Xu *et al.* 2003). In addition, cancer cell invasion has been shown to be dependent on Arf6 (Tague *et al.* 2004). Arf6 regulates a novel recycling pathway that is independent of clathrin-mediated endocytosis (Radhakrishna and Donaldson 1997). At least β1 integrin and MHCI in HeLa cells have been shown to use this recycling pathway (Brown *et al.* 2001). Expression of the activated Arf6 mutant (Q67L) promotes the accumulation of β1 integrin and MHCI in large vacuoles. Coincident with this event the cell rounds up and become symmetric, lacking polarity (Santy 2002), suggesting that the formation of cell polarity is coupled to recycling of membranes. Disruption of the actin cytoskeleton by cytochalasin D promotes the formation of large tubular structures containing actin, Arf6, β1 integrin, MHCI, and PIP5K (Brown *et al.* 2001). These tubular structures are believed to represent recycling membranes that are incompetent to fuse with the plasma membrane after they leave the recycling compartment (Brown *et al.* 2001). Because cytochalasin D interferes with the polymerization of actin it is clear that the Arf6 membrane pathway is closely associated with an actin-mediated process. Arf6 has also been shown to regulate adherens junction disassembly in epithelial cells (Palacios *et al.* 2001, Palacios *et al.* 2002). Hepatocyte growth factor (HGF) induces activation of Arf6 leading to redistribution of E-cadherin from cell junctions to early endosomes in MDCK epithelial cells. In this system, expression of a dominant-negative mutant of Arf6 (T27N) inhibits internalization of E-cadherin, thereby stabilizing the epithelial phenotype and blocking cell motility (Palacios *et al.* 2001, Palacios *et al.* 2002).

Arf GAPs, a set of multidomain proteins with Arf-GAP activity, have been shown to interact with actin-regulating proteins as well as with integrin-binding proteins (de Curtis *et al.* 2001). In addition, they affect Rac-mediated protrusive activity and cell migration. Multidomain Arf GAPs, possibly recruited by Rac, thus provide a possible link of Arf6-membrane endocytosis to sites of actin polymerization and may serve to coordinate membrane traffic and cytoskeletal reorganization during cell migration. Finally, Arf6-specific GEFs, like ARNO and EFA6, have been demonstrated to mediate

changes both in actin dynamics and membrane recycling (Santy and Casanova 2001, Franco *et al*. 1999).

### 1.7 Rab8

There are two Rab8 molecules in mammals; Rab8A and Rab8b. Very little is known about Rab8 function and its interacting proteins. The two Rab8 proteins are highly homologous and differ only in the hyper-variable C-terminus. Rab8A is expressed rather ubiquitously with the highest level of expression in muscle, lung and kidney whereas Rab8b is most abundant in brain, spleen and testis (Armstrong *et al.* 1996). Rab8A as well as Rab8b, on the single cell level have been found mainly on the plasma membrane and on vesicles in the cytoplasm when the expression level has been higher (Armstrong *et al.* 1996). Rab8 shows high sequence homology with Sec4 from *S.cerevisiae* (Huber *et al.* 1993a) and Ypt2p of *S.pombe* (Craighead *et al.* 1993).

The lipid modification essential for interaction with membranes and for biological function is in the case of Rab proteins a geranylgeranylation of one or two cystein residues at the C-terminus. Most Rab proteins have a -XXCC, -XCXC, or -CCXX lipid modification-motif at their C-terminus. Rab8 is one of a few Rabs that instead have a -CAAL motif. Rab8 is therefore geranylgeranylated only once, instead of the double prenylation of most other Rabs (Casey and Seabra 1996, Wilson *et al*. 1998). Although Rab8 is a substrate for GGTaseI (geranylgeranyltransferase type I) in cell-free assays, the majority of Rab8 is prenylated by the REP/GGTaseII system *in vivo,* like other Rabs (Wilson *et al.* 1998).

Only a few molecules have so far been shown to interact specifically with Rab8, two of these are Rab8ip and Trip8b. Rab8ip is a kinase, having sequence homology with GCK, which interacts with the GTP-bound form of Rab8 and is implicated in stress responses (Katz *et al.* 1994, Ren *et al.* 1996). Trip8b is a membrane receptor protein, interacting with Rab8b independently of which nucleotide bound and prenylation state (Chen *et al.* 2001). Another Rab8 interacting protein is Mss4, a small zinc binding protein that binds several Rabs, and can facilitate dissociation of GDP on them (Horiuchi *et al*. 1997). Mss4 is however not a true GEF for Rabs since it does not stimulate binding of GTP (Nuoffer *et al*. 1997). Recently, the first protein with GAP activity on Rab8 was found. This protein, the Akt substrate of 160 kDa (AS160) has been shown to have GAP activity for several Rabs including Rab8A (Miinea *et al*. 2005).

No difference in function between Rab8A and Rab8b has yet been detected. Co-expressed Rab8A and Rab8b have been shown to co-localize on vesicles in HeLa cells (Peränen and Furuhjelm 2001), indicating that they control the same membrane transport pathway. Furthermore, both proteins have a profound effect on cell shape and actin cytoskeleton organization (Peränen and Furuhjelm 2001). Rab8 was first shown to regulate the transport of vesicles from the trans-Golgi network to the basolateral surface of polarized epithelial cells (Huber *et al.* 1993a) and to dendrites in nerve cells (Huber *et al.* 1993b). It has, however, only a minor effect on transport kinetics of membrane transport from the Golgi-compartment to the plasma membrane, casting doubt to whether Rab8 in fact directly controls this pathway.

Activation of Rab8 induces polarized membrane transport of newly synthesized proteins to cell extensions (Peränen *et al*. 1996), and activation of Rab8 has also been found to miss-sort VSV-G to the apical surface domain in MDCK cells while the dominant negative mutant of Rab8 had no effect on sorting (Ang *et al*. 2003). Also in photoreceptor cells Rab8 has been shown a role in delivery of membrane from the TGN to specific sites at the plasma membrane (Deretic *et al.* 1995, Moritz *et al.* 2001). Furthermore Rab8 has recently been shown to regulate the transport of rhodopsin transport carriers (RTP) to the rod outer segment in photoreceptor cells (Deretic *et al*. 2004). These findings strongly indicate a key role for Rab8 in controlling membrane transport from the TGN to specific sites at the plasma membrane.

Interestingly, expression of Rab8 has a great impact on cell shape, which it achieves by reorganizing both actin and microtubules. In fact, Rab8A and Rab8b expression has been shown to contribute in the formation of membrane protrusions by reorganizing both the actin and the microtubule cytoskeleton in different cells (Armstrong *et al.* 1996, Peränen *et al.* 1996, Chen *et al.* 2001). These findings indicate an important role for Rab8 in the control of cell morphogenesis and polarity. In line with this, a role for Rab8 is emerging in neurite outgrowth and development of photoreceptors. A mutant Rab8 has been shown to cause cell death of transgenic Xenopus rods (Moritz *et al.* 2001) and depletion of Rab8 inhibits neurite outgrowth (Huber *et al.* 1995). Furthermore, mutations in optineurin, a Rab8 interacting protein, cause primary open-angle glaucoma (Rezaie *et al.* 2002) and in ADPKD (autosomal dominant polycystic kidney disease) cell loss of polarity is associated with redistribution of Rab8 (Charron *et al.* 2000).

# 2. AIMS OF THE PRESENT STUDY

Although there is a lot of information published about cell polarization, cell migration, and the molecular processes important for these functions, there is still very little known about the exact signals and mechanisms leading to cell polarization. Specifically, the role of membrane transport has been neglected to a large extent. The small GTPase, Rab8, is involved in polarized membrane transport and regulation of cell morphogenesis through reorganization of both actin and microtubules (Peränen *et al.* 1996). We decided to examine more closely the role of Rab8, its interacting proteins and its potential cross-talk with other small GTPases. In this way we hoped not only to learn more about the function of Rab8, but also about processes like cell polarization and cell migration.

Specific aims of this study were to:

1) **Find Rab8-activators i.e. GEFs, by searching for polypeptides interacting specifically with GDP-Rab8.**
I cloned the full-length sequence of one of the polypeptides found by two-hybrid screening: a novel protein with Rab8-GEF activity, that I have named Rabin8, and studied its interaction pattern and function in more detail.

2) **Find effector or linker proteins, by searching for polypeptides interacting specifically with GTP-Rab8.**
Out of the several interacting proteins found by two-hybrid screening, I first chose FIP-2 for cloning and further study. FIP-2 is a protein that links Rab8 to the Huntingtin protein. In addition, the interaction pattern of a further protein found in the same screen, JFC1, is investigated in publication III.

3) **Study the localization pattern of Rab8 in the cell.**
Compartmentalization of Rab8 has not been convincingly demonstrated to date. We therefore wanted to study the localization of Rab8 in the cell in relation to a number of indicator proteins and in this way learn more about its function.

4) **Investigate cross-talk between Rab8 and other small GTPases.**
By co-expression studies we have attempted to elucidate cross-talk between Rab8 and other small GTPases.

# 3. MATERIALS AND METHODS

The experimental methods used in this Ph.D.-thesis are listed in Table 2. Detailed descriptions of the methods can be found in the original publications.

**Table 2. Experimental methods used in this study**

| METHOD | PUBLICATION |
|---|---|
| General DNA methods | I, II, and III |
| Plasmid construction | I, II, and III |
| Western blotting | I, II, and III |
| Northern blotting | II |
| The yeast two-hybrid system | I, II, and III |
| Lambda triplex | II |
| Expression/purification of recombinant proteins | I, II and III |
| The NusA-protein expression system | II |
| In vitro and in vivo binding assays | I, II and III |
| GDP/GTP-exchange assays | II |
| Cell culture and transient transfections | I, II, and III |
| Stable cell lines | III |
| Antibodies, production of antisera and affinity purification | I, II, and III |
| Immunocytochemistry | I, II, and III |
| Cholera toxin and transferrin uptake | III |
| siRNA transfection | III |
| Time-lapse video microscopy | III |

# 4. RESULTS AND DISCUSSION

## 4.1 Yeast two-hybrid screens

In the first screen Rab8b was used as bait, lacking the lipid modification sequence and locked in the GDP-conformation. The library chosen was a human brain cDNA ready-cloned in the appropriate plasmid. The reason for this choice of library was that brain is one of the tissues where Rab8b has been found in highest abundance. The yeast was transformed with the bait and prey and 10 million clones were collected. These were tested for the reporter genes and about 40 colonies were isolated. The plasmid DNA was then isolated from these clones and the cDNA inserts tested by restriction digest to find groups of clones with a similar pattern meaning the inserts contained the same DNA sequence. Representatives of the groups of clones were then re-tested in the two-hybrid system to check that the correct plasmid was isolated and then if positive sequenced. Three groups of clones were found that fitted these criteria. They interacted specifically with the GDP-form of Rab8A as well as Rab8b, but not their GTP-form. The largest group consisted of Mss4, a protein previously described as a GDP-dissociation stimulator for various Rab proteins (Burton *et al.* 1994), indicating that we had found true interactors of Rab8. The other two groups consisted of closely related protein sequences, homologous to a previously described protein from rat by the name of Rabin3 (Brondyk *et al.* 1995). Rat Rabin3 interacts specifically with Rab3A and is a protein of unknown function. I isolated the full-length cDNA of one of our Rabin-homologues and named it Rabin8. This protein was found to interact with Rab8A and Rab8b in the two-hybrid system specifically in the GDP-bound conformation, and with Rab3Awt as well as GDP-bound Rab3A. As expected it did not interact with Rab8A, Rab8b, or Rab3A in their GTP-bound conformations.

In the second screen Rab8Awt was used as bait, lacking the C-terminal sequence for lipid modification. The library chosen was a human kidney cDNA cloned in the appropriate plasmid. The reason for the change of library was that Rab8A has a higher expression in kidney than brain. Out of the 1.2 million clones screened, a few hundred colonies were found expressing the reporter genes. These were grouped using hybridization and the cDNA inserts were simultaneously isolated and checked for the restriction-digest pattern. Several groups of clones were found. Representative clones were tested back in the two-hybrid system and clones interacting specifically with the GTP-form of Rab8, but not the GDP-form, were sequenced. Among the polypeptides found were sequences identical to proteins found in the gene bank, two of these FIP-2 and JFC1.

## 4.2 FIP-2 (Publication I)

### *4.2.1 A coiled-coil protein interacting with Rab8-GTP*

Some polypeptides discovered in the two-hybrid screen, interacting with Rab8 specifically in the GTP-bound form, were found identical in nucleotide sequence with

a protein in genebank called FIP-2 (Li *et al*. 1998). FIP-2 is a coiled-coil protein that is related to the NEMO protein, therefore also termed NRP (NEMO Related Protein). Further adding to the terminology confusion, a protein with identical sequence has also been named optineurin (Rezaie *et al.* 2002). Although not an apoptosis-inducing protein itself, FIP-2 interacts with the adenoviral E4-13.7 protein and prevents this adenoviral protein from inhibiting apoptosis due to NF-KB activation (Li *et al.* 1998). Mutations in the optineurin gene were identified as the cause for adult-onset primary open-angle glaucoma and the authors speculate that optineurin could have a neuroprotective function (Rezaie *et al.* 2002).

The full length cDNA of FIP-2 was cloned by PCR from human kidney cDNA and further tested in the two-hybrid system against a panel of different proteins. Full length FIP-2 interacted with Rab8A and Rab8b in the GTP-bound form and with wild type Rab8A and Rab8b but not with the GDP-bound form of either Rab8A or Rab8b. FIP-2 did not interact with either Rab2 or Rab3A, or with the negative control laminin provided with the system. These results show that the interaction of Rab8 with FIP-2 is highly specific and is likely to be relevant *in vivo*. I produced FIP-2 as a GST-fusion protein in order to test the binding of Rab8 and FIP-2 by GST-pulldown assay, but unfortunately both Rab8 and FIP-2 were insoluble when expressed in *E.coli.* However, a deletion mutant of FIP-2 was soluble as GST-fusion. Beads coupled to GST-FIP-2D were able to pull down *in vitro* translated GTP-Rab8 but not GDP-Rab8, whereas beads with GST alone did not pull down *in vitro* translated Rab8 in either form. These results reinforce the two-hybrid results showing a specific interaction between activated Rab8 and FIP-2. We also showed that the GTP-bound form of Rab8 is the preferred *in vivo* binding partner for FIP-2. This was tested by co-transfection of FIP-2 with his-tagged Rab8 mutants locked in either the GTP bound form (His-Rab8-Q67L) or the GDP-bound form (His-Rab8-T22N). Post-nuclear fractions of these cells were chemically cross-linked and then bound to Talon resin, which binds the His-tagged Rab protein. Substantially more FIP-2 bound to His-Rab8-Q67L beads than to the His-Rab8-T22N beads.

#### *4.2.2 FIP-2 links Rab8 to the Huntingtin protein*

A polypeptide found in the gene bank, shown to interact with the N-terminal region of the Huntingtin protein (Faber *et al.* 1998), has sequence identity with a part of the FIP-2 sequence. I was unable to clone full length huntingtin but instead managed to clone a polypeptide containing the first 555 amino acids of Huntingtin. This construct was tested in the two-hybrid system against full length FIP-2. I found that FIP-2 interacts with Huntingtin (1-555), but that Rab8 does not. In order to map the regions of FIP-2 important for binding to Rab8 and Huntingtin, I constructed deletion mutants and tested these in the two-hybrid system for interaction. Huntingtin (1-555) and Rab8 seemed to interact with separate regions of FIP-2. Whereas Rab8 interacts with a region in the N-terminus of FIP-2, Huntingtin interacts with a region in the C-terminus. A triple staining of transformed cells show partial co-localization of Rab8, FIP-2, and Huntingtin and although I have not been able to show a simultaneous interaction of the three proteins it is still an intriguing possibility.

In most cell types examined, FIP-2 has been shown to localize to the Golgi apparatus (Schwamborn *et al.* 2000, Stroissnigg *et al.* 2002). We also saw a partial co-localization of endogenous FIP-2 with the Golgi marker p115 in HT1080 cells (Fig 5, unpublished). In mature chicken erythrocytes FIP-2 was instead found in the marginal band (Stroissnigg *et al.* 2002). The marginal band is a thick ring-shaped microtubule bundle believed to perform a structural role in erythrocytes. We did not test the possibility of a direct interaction between FIP-2 and microtubules. However, Hoffner *et al.* (2002) have shown that Huntingtin, which binds FIP-2, specifically interacts with β-tubulin and binds to microtubules. The perinuclear localization of Huntingtin was shown to be a consequence of this interaction (Hoffner *et al.* 2002). Recently, FIP-2/optineurin has been shown to be essential for the structure of the Golgi complex (Sahlender *et al*. 2005). When FIP-2 was depleted from cells by RNAi, the Golgi complex was fragmented into vesicular and short tubular structures, dispersed throughout the cytoplasm. The overall structure of the fragments was preserved, but the ribbon structure of Golgi was lost. The morphological changes observed were similar to those seen when microtubules are disrupted by drugs such as nocodazole, suggesting that FIP-2 may link Golgi membranes, directly or indirectly, to microtubules around the MTOC (Sahlender *et al*. 2005).

### *4.2.3 FIP-2 and vesicle transport*

The intracellular localization of Huntingtin has implicated it in vesicle transport (DiFiglia *et al.* 1995, Velier *et al.* 1998). Our finding that Huntingtin is indirectly linked to Rab8 strongly supports this. In addition, Huntingtin has also been directly implicated in vesicle transport of neurotrophic factors along microtubules (Gauthiere *et al*. 2004).

A similar intracellular distribution of Huntingtin (Velier *et al.* 1998) and Rab8 (Peränen *et al.* 1996, Huber *et al.* 1993a) (to the plasma membrane, the trans-Golgi network and to vesicles in the cytoplasm), indicates that these proteins may interact directly or indirectly. We wanted to determine how FIP-2, Huntingtin and Rab8 affect the localization of each other in cells. We found that FIP-2 was able to redistribute Huntingtin from the cytoplasm onto vesicular structures and that Rab8-Q67L also co-localizes with FIP-2. We believe that Rab8 can recruit Huntingtin to these vesicular structures with the help of endogenous FIP-2. Recently Sahlender *et al*. (2005) has suggested a similar complex, linking Rab8 to myosinVI via FIP-2/optineurin. The authors show that the tail of myosin VI binds directly to optineurin and suggest that this interaction links the small GTPase Rab8 to the actin based motor protein myosinVI.

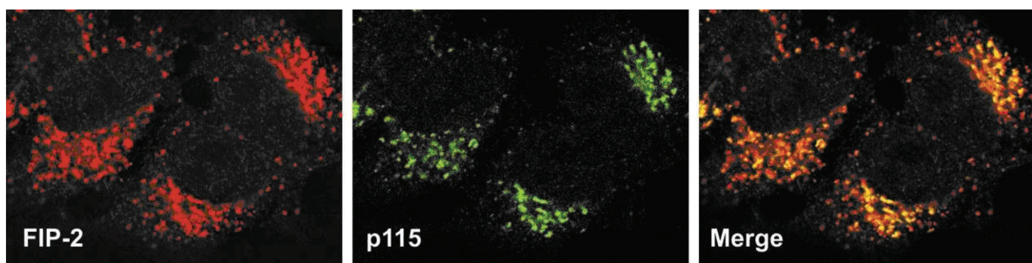



**Fig 5. Co-localization of endogenous FIP-2 with the Golgi marker p115.** (Peränen, unpublished)

*4.2.4 FIP-2 and Rab8 both affect cell morphogenesis*

Expression of Huntingtin (1-555) did not markedly change cell shape whereas moderate expression of FIP-2 led to formation of extended lamellar and tail structures. This phenomenon was inhibited by expression of Rab8-22N suggesting that FIP-2 may act upstream of Rab8. Interestingly, another Huntingtin binding protein, HAP-1, has been shown to promote neurite extension in PC12 cells (Li *et al.* 2000). This together with the fact that FIP-2 also affects cell shape implicates Huntingtin in processes controlling cell morphogenesis. HAP-1 links Huntingtin to dynactin, suggesting that Huntingtin could be part of a protein complex needed for the motility of membrane vesicles along microtubules (Engelender *et al.* 1997). In addition, HAP-1 binds a Trio-like polypeptide with a Rac1 GEF-domain that is involved in actin dynamics (Colomer *et al.* 1997). Huntingtin also interacts with HIP1, a protein that links membrane vesicles to the actin cytoskeleton (Wanker *et al.* 1997). These facts along with the findings that FIP-2 associates with the myosinVI motor protein (Sahlender *et al.* 2005) implicate Rab8 in rearrangements of both actin and microtubules.

The fact that FIP-2 expression can be induced by TNF-$\alpha$ (Li *et al.* 1998) is interesting because Rab8 also binds another protein Rab8ip/germinal center kinase (GCK), which is activated by TNF-$\alpha$ (Ren *et al.* 1996). One role for Rab8 could thus be in mediating responses to stress. Some evidence implicates an important role for Huntingtin in apoptosis (Zeitlin *et al.* 1995, Rigamonti *et al.* 2000). FIP-2 interacts with the adenoviral protein E3-14.7K protein, which protects cells from apoptosis induced by TNF-$\alpha$. E3-14.7K and Huntingtin bind to a C-terminal region of FIP-2 raising the possibility that binding of Huntingtin to FIP-2 could mediate apoptotic signals. Interestingly, recently it was shown that optineurin/FIP-2 increases cell survival and that this is associated with translocation of optineurin into the cell nucleus in a Rab8-dependent manner upon an apoptotic stimulus (De Marco *et al.* 2006). The fact that expression of mutant Huntingtin increases the expression of several inflammatory-related mRNAs (Luthi-Carter *et al.* 2000) also supports this hypothesis.

## 4.3 Rabin8 (publication II)

I have identified a GEF, specific for Rab8 that is recruited onto vesicles and is translocated possibly along actin filaments to dynamic actin-containing protrusions at the cell periphery. This process can be induced by phorbol esters and leads to cell polarization by modulating actin organization.

*4.3.1 A coiled-coil protein interacting with Rab8-GDP*

Several polypeptides with high sequence homology to a known protein from rat called Rabin3 were found in the two-hybrid screen for proteins interacting with the GDP-mutant of Rab8b (T22N). Rat Rabin3 is a protein of unknown function that interacts specifically with Rab3A in its GDP-bound or nucleotide-free state (Brondyk *et al.* 1995). None of the clones contained the full-length cDNA of the human gene, so a commercial

human-brain lambda-triplex cDNA library was screened for the full-length sequence. A PCR-based method was used to isolate candidate phages and finally hybridization was performed to identify the positive phages. Two independent phages were picked and the cDNAs were isolated and then sequenced. Both clones contained the full-length cDNA of the protein we have named Rabin8, the same CDS has since been entered into the gene bank by others (NCBI; accession # BC002556). Rabin8 was tested in the two-hybrid system against a panel of different proteins and was found to interact specifically with Rab8A and Rab8b in the GDP-bound form and with Rab3A more strongly in the GDP-bound form than with wild-type. It does not interact with the more distantly related Rab2 protein, indicating that the interaction of Rabin8 with Rab8 is highly specific. These results were confirmed by other *in vitro* binding studies. *In vitro* binding experiments also showed that Rabin8 has the potential to form homodimers or multimers.

I constructed a number of deletion mutants of Rabin8 to find the region(s) needed for binding of Rab8. I found that neither the N-terminal half (aa1-221) nor the C-terminal half (aa222-460) was able to bind Rab8-T22N in the two-hybrid system. However, the 1-316aa and 101-316aa constructs were able to bind the GDP-bound forms of Rab8A and Rab8b. The Rabin8 (101-316)-construct, able to bind Rab8-T22N, contains the entire coiled-coil domain (aa149-244) as well as a potential RhoA-binding HR1 motif. We were not able, however, to show any interaction between Rabin8 and RhoA either in two-hybrid or *in vitro*-binding assays (Hattula and Peränen, unpublished).

### *4.3.2 Rabin8 is a Rab8-specific GEF*

The expression of Rabin8 was examined by Northern blot analysis of several adult human tissues. It was found to have a similar expression pattern as in rat for Rabin3. The mRNA was detected in all tissues tested with the highest level of expression in brain, heart, and kidney. The signal was very weak possibly suggesting that the message was low in abundance. To obtain Rabin8-specific antibodies we produced recombinant Rabin8 as a his-GST fusion protein from the pGAT2 vector, and antibodies to this fusion protein were raised in rabbits. Affinity purified anti-Rabin8 antibodies were used to detect Rabin8 from the lysates of several cell lines (HeLa, Jurkat, A431, and endothelial cells). A band of the appropriate size, 50kDa, was detected in all four cell-lines with the highest abundance in Jurkat and A431 cells and the lowest in endothelial cells.

When expressed in cells Rabin8 was found localizing to the plasma membrane and in some cells it induced the formation of processes resembling those seen in cells expressing activated Rab8, indicating that Rabin8 expression activates Rab8 *in vivo*. In GDP/GTP-exchange assays recombinant NusA-Rabin8 was then shown to increase the rates of GDP release as well as GTP binding on recombinant Rab8, but not on Rab3A or Rab5, showing that Rabin8 is an exchange factor specific for Rab8. When rat Rabin3 was cloned and expressed as recombinant NusA-Rabin3 we, like the original authors (Brondyk *et al.* 1998), could not show an exchange activity towards Rab3A. We did, however, show a clear GDP/GTP-exchange activity with Rab8 as substrate. Our conclusion therefore is that also the rat Rabin3 is a GEF specific for Rab8.

Only a few Rab GEFs have so far been identified. Two of these, Rabex-5 (Horiuchi *et al.* 1997) and RIN (Tall *et al.* 2001), are both exchange factors for Rab5. Rab3-GEP,

shows GEF activity towards Rab3A, Rab3C, and Rab3D but not Rab3B (Wada *et al.* 1997) and a Rab3-GEP homologue, AEX-3 has been found in *Caenorhabditis elegans* (Iwasaki and Toyonaga 1997). None of these GEFs show sequence identity with Rabin8. In yeast, however, Sec2, the exchange factor for the Rab8 homologue Sec4, does show some identity with Rabin8 (Walch-Solimena *et al.* 1997), as does the Rab3-specific GEF, GRAB (Luo *et al.* 2001). Rabin8 and GRAB show an overall sequence homology of 60%, and their coiled-coil domains, which in both cases are used for Rab-binding, are almost identical. It has also been shown that GRAB binds inositol hexaphosphate kinase (InsP6K1) through the same coiled-coil region (Luo *et al.* 2001), suggesting that this may also be the case for Rabin8. However, we have not tested this.

### *4.3.3 Rabin8 is closely associated with actin structures*

HeLa cells transfected with Rabin8 had less stress fibers than the untransfected cells and showed an increase in ruffles and protrusions at the cell surface. Rabin8 was shown to co-localize with actin in these structures. Disruption of microtubules by treatment with nocodazole led to the formation of strong stress fibers. In such cells Rabin8 was detected uniformly along the cell membrane rather than being found co-localizing with actin. The RhoG-specific GEF, TrioD1, has shown a similar sensitivity to microtubule depolymerization (Blangy *et al.* 2000). Treatment of Rabin8 transfected cells with cytochalasin D, disrupting the cortical actin, led to Rabin8 being found in patches together with actin. In yeast, the Sec4 GEF, Sec2, has been shown to be dependent on actin for its localization (Elkind *et al.* 2000). Although we could not detect a direct interaction between Rabin8 and purified actin *in vitro*, our results still indicate that Rabin8 is dependent on microtubules for proper function and is closely associated with the actin cytoskeleton. The association with actin is likely to be indirect, possibly mediated by myosins. Interestingly, Rab8 has been shown to co-localize with the tail of myosin-Vc when expressed in HeLa cells (Rodriguez and Cheney 2002) and FIP-2, another Rab8 interacting protein, has been found to bind to the tail of myosin VI (Sahlender *et al*. 2005). Alternatively, Rabin8 could bind a lipid or a lipid-binding protein at the plasma membrane that participates in actin dynamics (Lanier and Gertler 2000).

Phorbol esters activate PKC and modulate actin assembly. Treatment of HeLa cells with phorbol esters, thereby activating PKC, leads to loss of stress fibers and the appearance of actin-containing protrusions, resembling lamellipodia (Frank *et al.* 1998). In addition, phorbol esters regulate the expression of the Rab8-interacting protein, FIP-2 (publication I, Schwamborn *et al.* 2000). In view of all this along with our observation that Rabin8 seems to be closely associated with the actin cytoskeleton, we wanted to examine the effect of phorbol ester on cells expressing Rabin8. Treatment of Rabin8-transfected cells with PMA (phorbol ester) lead to a dramatic redistribution of both actin and Rabin8 to the periphery of lamellipodial structures. ARNO, a GEF for Arf6, has also been shown to re-localize in a similar way in cells treated with phorbol ester (Frank *et al.* 1998). Rab8 specific vesicles in cells treated with PMA also redistributed from the perinuclear region to a location close to the Rabin8 positive area. These results show that activation of PKC promotes a polarized distribution of Rab8 and Rabin8 to actin-containing structures at the cell surface, and indicates that the polarization of Rab8 vesicles to the cell periphery is linked to Rabin8's translocation.

### *4.3.4 Rabin8 mediates polarized membrane transport of Rab8-specific vesicles*

In cells co-transfected with dominant negative Rab8-T22N and Rabin8 we could see re-localization of both Rab8-T22N and Rabin8 to intracellular vesicles. These vesicles could sometimes be seen in rows along actin fibers, possibly traveling along these filaments to the cell surface where the vesicles accumulate at the tips of actin containing protrusions. Rabin8 seems to neutralize the negative effect of dominant negative Rab8-T22N and promoted the formation of cell protrusions, perhaps by activating endogenous Rab8. Since co-transfection of Rabin8 with dominant active Rab8-Q67L did not result in redistribution of Rabin8 from the plasma membrane onto vesicles, the recruitment of Rabin8 must be dependent on GDP-Rab8.

A mutant Rabin8, lacking the C-terminal end, that is still able to bind Rab8, was co-transfected with dominant negative Rab8-T22N. The result was that the cells no longer became polarized. Vesicles positive only for Rab8 were seen in the perinuclear region and not at the cell periphery where the mutant Rabin8 was found. The co-localization on intracellular vesicles observed with Rab-T22N and full length Rabin8 was abolished in these cells. This implies that Rabin8's C-terminal end is essential for the recruitment of Rabin8 onto Rab8 vesicles and is also necessary for the Rab8 vesicles to be targeted to the plasma membrane. The targeting could possibly be achieved by way of Rabin8 binding to a membrane bound receptor, as has been suggested for Sec2 (Elkind *et al.* 2000). Interestingly, a protein called Elp1 was recently shown to bind to the C-terminus of Sec2p, the yeast homolog of Rabin8 (Rahl *et al*. 2005). Furthermore, the Sec2p interaction domain of Elp1 is necessary for the function and localization of Sec2p. Whether the human homolog of Elp1, IKAP, is necessary for Rabin8 function is not known.

Could Rab GEFs have roles other than to simply activate their Rab proteins? Rabin8 is a coiled-coil protein that has the potential to self-associate. It does not appear to bind actin directly but it is nonetheless closely associated with cortical actin. Rabin8 could achieve this by binding an actin-binding protein like myosin or perhaps a lipid or lipid-binding protein at the membrane participating in actin dynamics. Vesicular Rabin8 could bind an actin associated Rabin8 at the plasma membrane, and Rabin8 self-association could promote vesicle movement along actin filaments to an appropriate site for fusion. It is not known whether Rabin8 is needed for the initial transport of Rab8 vesicles along microtubules to actin filaments. Rabin8 might activate Rab8 at several points during transport to the cell surface, and could allow for the recruitment of different Rab8 effectors during vesicle generation, movement and fusion or alternatively other Rab8-specific GEFs may be involved in these processes.

## 4.4 Characterization of the Rab8-specific membrane traffic route linked to protrusion formation (Publication III)

### *4.4.1 Rab8 affects cell polarity.*

The membrane traffic route regulated by Rab8 is still somewhat unclear. Several reports though, indicate an important role for Rab8 in the regulation of cell morphogenesis and cell fate. Expression of Rab8 has a big impact on cell shape due to reorganization of the

actin and microtubule cytoskeleton (Armstrong *et al.* 1996, Peränen *et al.* 1996, Chen *et al.* 2001). Also the Rab8-interacting proteins Rabin8 and FIP-2/optineurin modulate cell morphogenesis (publication II, publication I, Li *et al.* 1998). Mutant Rab8 has been shown to cause cell death of transgenic Xenopus rods (Moritz *et al.* 2001), and depletion of Rab8 has been shown to inhibit neurite outgrowth (Huber *et al.* 1995). Furthermore, mutations in optineurin/FIP-2 cause primary open-angle glaucoma (Rezaie *et al.* 2002)

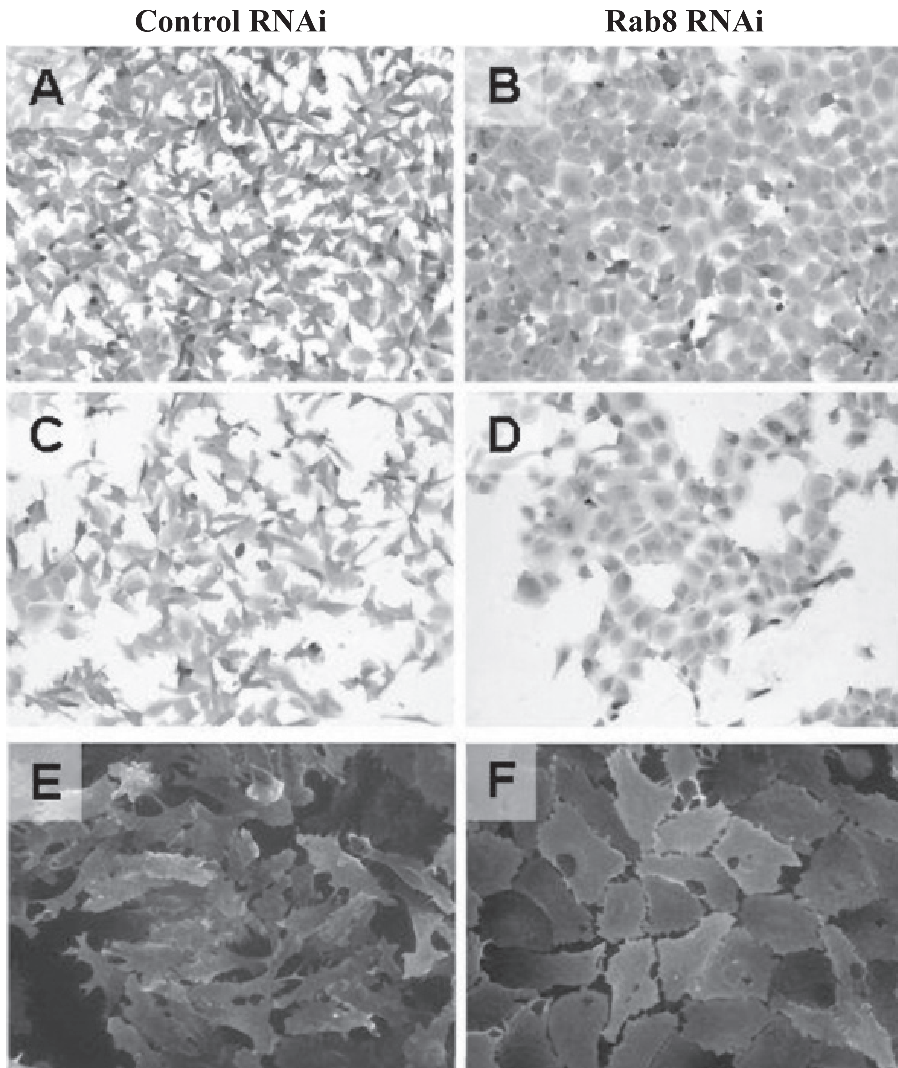



**Fig. 6. Rab8 depletion promotes cell-cell adhesion and leads to loss of cell polarity.** (This figure is a composite of Fig.1 and Suppl. Fig.1, publication III). The cells were either transfected with a control RNAi (A, C, E) or with a Rab8-specific RNAi (B, D, F). Shown in A-D are HT1080 fibrosarcoma cells where a marked difference in cell shape and cell-cell contact can be noted. The cells in E and F illustrate the same phenomenon at a higher magnification, only they are HeLa cells stained with anti-transferrin receptor. Cells transfected with the control RNAi are elongated with protrusions, while cells depleted of Rab8 are more symmetric and form close cell-cell contacts.

and in ADPKD (autosomal dominant polycystic kidney disease) cell loss of polarity is associated with redistribution of Rab8 (Charron *et al.* 2000).

In order to discover more about Rab8 function in cells, we created stable cell lines (HT1080 fibrosarcoma cells) expressing Rab8 constitutively active (Q67L) and dominant negative (T22N), fused to the C-terminus of enhanced green fluorescent protein (EGFP). These cell lines were compared by microscopy to control cells stably transfected with the expression vector (pEGFP-C1). The cells expressing Rab8-T22N lost their polarity, acquired a cubical organized structure, and grew closely together, in a way resembling epithelial cells. Importantly, depletion of Rab8 by siRNA also promoted cell-cell adhesion and led to inhibition of cell protrusions (Fig. 6). In contrast, the cells expressing Rab8-Q67L were very spiked in appearance with long protrusions and seemed to have lost contact inhibition; at times it looked like they could crawl on top of each other. Together, this suggests that Rab8 is essential in regulating cell protrusion, cell-cell adhesion and polarity.

In publication II, we showed that endogenous Rab8 exhibited a polarized localization to the tips of protrusions in HeLa cells and was often found in filopodia. We discovered that some cells lacked a polarized distribution of Rab8 and decided to test whether this was due to cell density. We found that cells plated in low density had a very high ratio of polarized Rab8 whereas cells plated confluent had very little polarized Rab8. Dominant negative Rab8-T22N localizes to the perinuclear region, similarly to endogenous Rab8 in non-polar cells (cells plated at high density). In contrast, the constitutively active Rab8-Q67L localizes to the cell periphery, the plasma membrane and tips of protrusions as does the endogenous Rab8 in polarized cells (cells plated at low density). Polarized distribution of endogenous Rab8 is indeed linked to cell density.

### *4.4.2 Rab8 compartmentalization*

No clear evidence has been presented on the compartmentalization of Rab8. In publication III, we show the distribution of Rab8 compared to several marker proteins. Constitutively active Rab8-Q67L (GFP- or myc-tagged) co-localizes with β1 integrin on intracellular vesicles and on large tubular structures. These tubules, about 10-20μm long, were often seen in cell protrusions. Dominant negative (GFP- or myc-tagged) Rab8-T22N, however, showed reduced co-localization with β1 integrin. Instead, it was found in a reticular region surrounding large vacuoles containing accumulated β1 integrin. This accumulation of integrin into vacuoles was associated with loss of cell protrusions and an increase in cell symmetry. Co-expression studies show that Rab8-T22N inhibits the tubular structures and cell surface extensions induced by Rab8-Q67L, indicating that the activity of the Rab8/β1 integrin-positive tubular membrane compartment is associated with formation of cell surface extensions. Often studying the localization of endogenous protein in cells is hampered by bad antibodies and low levels of targets. We have, however, raised an antibody that after affinity purification worked very well in paraformaldehyde fixed cells. Using this antibody we were able to show that endogenous Rab8 was found on the same vesicular and tubular structures as seen for expressed tagged versions of Rab8. Moreover, endogenous Rab8 was seen co-localizing with β1 integrin on tubular structures in several human cell lines eg. HeLa, Paju, and HT1080.

To test if Rab8 is linked to receptor-mediated endocytosis and/or recycling we looked at co-localization of endogenous Rab8 with GFP-tagged Rab4, Rab5 and Rab11 in HT1080 cells. Very little co-localization of endogenous Rab8 was seen with Rab4 and Rab5, while some co-localization was observed with Rab11. This co-localization was observed in a pericentriolar region (possibly corresponding to the endocytic recycling compartment), on vesicles at the leading edge and sometimes in the tips of protrusions. The partial co-localization of Rab8 with Rab11 points to a possible role for Rab8 in the trafficking of transferrin (Tfn) and the transferrin receptor (Tfn-R). We therefore proceeded to look at Tfn internalization and recycling in Rab8 depleted cells.

There was no difference in uptake of Tfn in Rab8 depleted cells compared with the control cells. However, in control cells Tfn accumulated in the pericentriolar region, whereas in Rab8-depleted cells Tfn-vesicles were randomly scattered in the cytoplasm. In both Rab8-depleted and control cells Tfn was then externalized normally. We also looked at the effect of expressing mutant Rab8-T22N or Rab8-Q67L. Expression of the dominant negative Rab8-T22N had the same effect as Rab8 depletion, that is, vesicles with Tfn were found scattered randomly instead of accumulating in the pericentriolar region as seen with the cells expressing the activated Rab8-Q67L (data not shown). Rab8 activity thus does not seem to be needed for internalization and recycling of Tfn but rather its delivery to the pericentriolar region.

Next we looked at the distribution of the Tfn-R in Rab8-depleted cells. In control HeLa cells Tfn-R was found accumulated in the pericentriolar region in about 60% of the cells, and in 30% of the cells Tfn-R co-localized with Rab8 in protrusions. In Rab8-depleted cells, however, Tfn-R was randomly located in vesicles throughout the cytoplasm. In these cells we hardly saw any accumulation of Tfn-R in protrusions. We therefore conclude that Rab8 activity is required for directing the Tfn-R to the pericentriolar region and to cell surface protrusions. In agreement with this Rab8 has been shown to be important for the polarized targeting of AMPA receptors to the spine surface and for AMPA receptor recycling at the post synaptic terminal (Gerges *et al*. 2004).

Rab8 and Rab11 thus seem to direct different aspects of Tfn/Tfn-R recycling. Rab11 controls the transport of Tfn from the endocytic recycling center (ERC) to the plasma membrane and Rab8 is instead needed for the delivery of Tfn/Tfn-R to the ERC (Ullrich *et al.* 1996, publication III). In Rab8-depleted cells Tfn is seen in small vesicles, probably representing sorting endosomes. Their recycling was largely unaffected by Rab8 depletion and is most likely regulated by a Rab4-dependent pathway. The role of Rab8 may be indirect, perhaps by regulating cytoskeleton-based movement of vesicles (Chabrillat *et al*. 2005, publication III). Overexpression of the tail of myosin-Vc has been shown to lead to colocalization of Tfn-R and Rab8, and to perturb Tfn trafficking (Rodriguez and Cheney 2002). Rab8 has furthermore been shown to regulate microtubule dynamics (Peränen *et al.* 1996). It is therefore feasible that one explanation for the random localization and transport of Tfn/Tfn-R in Rab8-depleted cells is for Rab8 to be involved in organization of the ERC and in facilitation of vesicle movement along microtubules from the ERC to cell surface domains.

We had observed that in stable cell lines expressing dominant negative Rab8-T22N the cells were symmetric in shape and had close cell-cell contacts, unlike the

cells expressing wt Rab8 or constitutively active Rab8-Q67L. We wanted to look at β-catenin in these cell lines to further assess the potential role of Rab8 in cell-cell adhesion. In Rab8-T22N transfected cells, β-catenin was found mainly at sites of cell-cell contacts (data not shown) correlating with the fact that Rab8-T22N seems to promote the formation of cell-cell contacts (Hattula and Peränen, unpublished). In Rab8-Q67L transfected cells though, cell-cell contacts were lost and β-catenin was instead found in a perinuclear region and at the tips of protrusions partially co-localizing with Rab8-Q67L (data not shown). Interestingly, expression of Rab8-Q67L in polarized epithelial cells (MDCK) also promoted redistribution of some β-catenin to Rab8-specific vesicles (Hattula and Peränen, unpublished). Moreover, these MDCK cells showed cell protrusions or extensions that had grown through the region between adjacent cells (data not shown). These findings indicate that Rab8 activation is associated with turnover of components of the adherens junction, and that Rab8 might be important in modulating cell polarity in situations where a rapid change in the cell shape is needed.

### *4.4.3 Cross-talk between Rab8 and RhoA*

Rab8 expression is associated with actin reorganization by exchanging actin stress fibers for actin-containing ruffles, lamellipodia and filopodia (Peränen *et al*. 1996, Peränen and Furuhjelm 2001). Since actin polymerization/depolymerization might influence the dynamics of Rab8-containing vesicles we went on to investigate its role by incubating cells with cytochalasin D, an actin depolymerizing drug.

Addition of cytochalasin D led to a dramatic increase in the number of cells containing tubular structures that stained for endogenous Rab8. Similar results were also seen upon latrunculin B addition and upon expression of EGFP-C3 exotransferase (data not shown). When cells transfected with RhoA-T19N were treated with cytochalasin D, there was still the same increase in tubule formation. Expression of RhoA-G14V, however, clearly inhibited the formation of these tubules. In these cells the tubules were replaced by vesicular structures that were found mainly in a perinuclear region. In cells expressing RhoA-T19N, not treated with cytochalasin D, we found that Rab8 was localized normally, accumulating in cell protrusions. In cells not treated with cytochalasin D instead expressing RhoA-G14V, Rab8 was redistributed to a perinuclear region. Here we also saw a slight reduction in number of cells containing tubules (not shown). We also showed that microtubules are essential for the formation of Rab8-specific tubules as virtually no tubules could be seen if cells were pre-treated with nocodazole, a microtubule depolymerizing drug, before addition of cytochalasin D.

Rab8 has been shown to promote polarized transport of the G protein of the vesicular stomatitis virus (VSV G) (Peränen *et al.* 1996). When cells expressing a GFP fusion of VSV G (VSVG3-GFP) were treated with cytochalasin D, Rab8 and VSVG3-GFP co-localized to patches on the plasma membrane where Rab8 tubules entered (Fig. 7). However, we seldom saw VSVG3-GFP in these tubules. Instead, GFP-vesicles could be seen following the same pathway as the Rab8-tubules, indicating that Rab8 indirectly promotes the polarized delivery of VSVG3-GFP vesicles (data not shown). This is also supported by the fact that Rab8 depletion by siRNA does not inhibit the traffic of VSVG3-GFP to the plasma membrane (Peränen, unpublished).

Altogether, these results demonstrate that actin polymerization and depolymerization, regulated by RhoA, in addition to microtubules have a critical role in the biogenesis of Rab8-specific tubular structures.

*4.4.4 Clathrin-independent pathways in Rab8 mediated processes*

Arf6, Rab22a and dynamin mutants have been shown to inhibit the transport of CTxB (Kirkham *et al*. 2005, Mesa *et al.* 2005). Rab8 colocalizes with Arf6, Rab22b, and somewhat with Rab22a, indicating that Rab8 together with these proteins could be involved in regulation of the clathrin-independent pathway through which CTxB is transported (Rodriguez-Gabin *et al.* 2001, publication III, Peränen unpublished). Trafficking of CTxB is furthermore actin-dependent as is the Rab8-specific recycling pathway (Badizadegan *et al*. 2004, publication III). Cholera toxin B (CTxB) enters the cell in different ways (Shogomori and Futerman 2001, Massol *et al.* 2004, Kirkham *et al*. 2005). It can for example bind to the glycosphingolipid GM1 through which it is then endocytosed via the Golgi complex to the ER (Kirkham *et al*. 2005).

We investigated the possible role for Rab8 in the transport of CTxB to the Golgi complex by applying Alexa-594 conjugated CTxB to cells depleted of Rab8 by RNAi and by comparing these to control cells treated in the same way. After a 30 min chase about 80% of the cells treated with control RNAi showed a Golgi-specific staining compared with only 15% of the cells treated with Rab8-specific RNAi. In the Rab8 depleted cells the CTxB was found on the plasma membrane and on small peripheral vesicles. However, a longer chase period increased the Golgi accumulation of CTxB in Rab8 depleted cells, suggesting that although Rab8 does not block the retrograde transport of CTxB it clearly slows it down. Consequently Rab8 is likely to function at a step between the plasma membrane and the Golgi complex.

Arf6 regulates membrane traffic between the plasma membrane (PM) and a non-clathrin derived endosomal compartment. Arf6-positive tubular structures, similar to the Rab8-specific tubules we have described here, have previously been shown to form upon treatment of cells with cytochalasin D (Brown *et al.* 2001). We saw that in cells expressing Arf6 wt, treated with cytochalasin D, these tubular structures stained positive

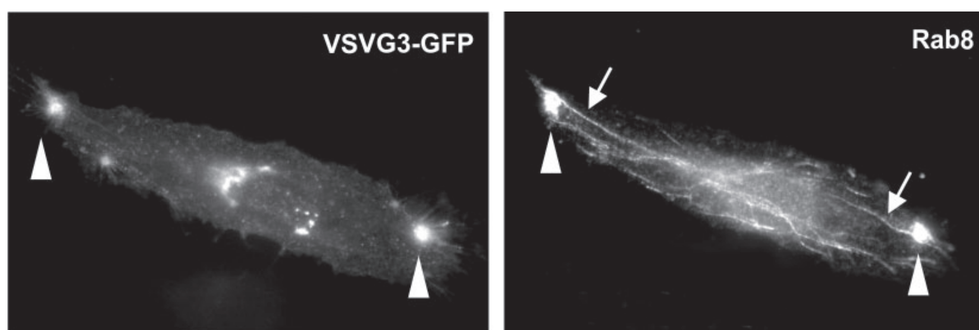



**Fig. 7. Distribution of endogenous Rab8 and VSV-GFP after cytochalasin D treatment.** Rab8 is found on tubular structures (arrows) that sometimes are connected to patches at the plasma membrane containing Rab8 and VSV-GFP (arrow heads). (Hattula and Peranen, unpublished.)

both for the recombinant Arf6 and endogenous Rab8. The tubules also contained β1-integrin, plakoglobin and β-catenin, as has been previously shown for Arf6-tubules (data not shown, Brown *et al.* 2001). Depletion of Rab8 by RNAi led to a clear decrease in the number of Arf6 tubules and instead a stronger staining of Arf6 on the plasma membrane (not shown). Furthermore while expression of dominant negative Arf6-T27N didn't affect the formation of Rab8-specific tubules in cells after treatment with cytochalasin D, expression instead of constitutively active Arf6-Q67L effectively blocked it. In these cells Rab8 tubules were absent or found fragmented as small vesicles.

Co-transfection of Arf6 wt with Rab8-Q67L led to re-localization of both Arf6 and Rab8 to tubules, seen entering lamellae in prominent cell surface extensions. Co-transfecting cells with dominant negative Arf6-T27N and Rab8-T22N led to cells lacking extensions and in these cells Rab8 and Arf6 co-localized on intracellular vesicles (not shown). Arf6-T27N partially inhibited cell surface extension induced by Rab8-Q67L and these two recombinant proteins were seen to co-localize on vesicles and tubules in cells where they were co-transfected. Cells transfected with activated Arf6-Q67L, were rounded and accumulated large vacuoles, as has been previously shown for other cell lines (Brown *et al.* 2001, Santy 2002). Co-transfection of Arf6-Q67L and Rab8-Q67L inhibited the Rab8 induced cell extension and re-located Rab8-Q67L to a perinuclear region, where Rab8-T22N is normally seen, indicating that Arf6 works upstream of Rab8. Taken together, our results clearly suggest that Arf6 and Rab8 are functionally linked in the control of recycling of membranes that participate in the formation of cell surface extensions.

### *4.4.5 JFC1 is a Rab8-specific effector*

As previously described (publication I), we used Rab8A wt as bait to screen a human kidney yeast two-hybrid cDNA library to look for novel Rab8 effector proteins. We found several identical clones that correspond to the open reading frame encoding the human JFC1 protein (McAdara-Berkowitz *et al.* 2001). JFC1 is a member of the synaptogamin-like protein (Slp) family, and has been shown to interact with Akt, Rab27a, and the NADPH oxidase (Johnson *et al*. 2005a, Johnson *et al.* 2005b, McAdara-Berkowitz *et al*. 2001).

In the yeast two hybrid system we found that JFC1 interacted with both mutants of Rab27a, GDP-bound T23N and GTP-bound Q78L, but not with negative controls Rab2-Q65L or laminin. In contrast to the result with Rab27a, JFC1 interacted highly specifically with Rab8; binding to Rab8 wt and GTP-bound Rab8-Q67L but not to GDP-bound Rab8-T22N. These results were followed up by *in vitro* binding studies, confirming the interaction of Rab8 and JFC1. In these assays the binding of JFC1 was both stronger and more specific to Rab8 than to Rab27a. Another set of experiments where GST-JFC1 was used to pull down Rab8 from cell extracts further strengthened the case for a highly specific interaction between JFC1 and Rab8. Recombinant GFP-Rab8-Q67L as well as endogenous Rab8 was efficiently pulled down by GST-JFC1, whereas recombinant GFP-Rab8-T22N was not. The fact JFC1 interacts with Rab27a as well as Rab8 suggests that these Rab proteins may functionally overlap. This is further supported by studies showing co-localization of Rab8 and Rab27a on dense-core vesicles

and melanosomes, and involvement of both Rabs in actin-dependent movement of the melanosomes (Chabrillat *et al*. 2005, Fukuda *et al*. 2002a). Moreover, Slp4-a, another member of the Slp family, has also been shown to bind both Rab8 and Rab27a (Fukuda 2003).

A co-transfection approach was used to show *in vivo* interaction between Rab8 and JFC1. In the transfected cells JFC1 is produced together with GST, GST-Rab8-T22N, or GST-Rab8-Q67L. Cell lysates from these cells were incubated with glutathione-Sepharose beads resulting in precipitation of JFC1 only by the GST-Rab8-Q67L protein. Thus, JFC1 clearly prefers Rab8-GTP as a binding partner also *in vivo*. We frequently saw co-localization of Rab8 with recombinant JFC1 in HeLa cells, an association that was enhanced by treatment of the cells with cytochalasin D. We also saw that expression of recombinant JFC1 increased the association of Rab8 with vesicles and tubules. Coincident with this we observed a decrease in the number of Rab8-specific tubules and an increase in the number of Rab8-specific vesicles and vacuoles. At the moment we do not know whether this is due to an inhibition of tubule formation or a promotion of the disassembly of existing tubules.

### *4.4.6 Rab8 mediates membrane recycling at the leading edge*

To gain a better understanding of the Rab8 transport route, we looked at GFP-tagged Rab8 proteins by time-lapse video microscopy in living cells. In HT1080 cells expressing GFP-Rab8-Q67L, Rab8-positive macropinosomes and vesicles are formed at ruffling areas at the leading edge and transported toward the cell center. In addition we could also see traffic of rapidly moving vesicles moving from the cell center toward the leading edge. The macropinosomes are closely connected with tubular structures and we could occasionally see smaller vesicles fuse with these tubules. We could furthermore see the tubules independently attach to and detach from the plasma membrane.

We next looked at recombinant GFP-Rab8b wt in motile live NIH3T3 cells. GFP-Rab8b was seen mainly at ruffling areas and on tubular structures forming at these areas. Most of these tubules moved inwards toward the cell center but sometimes tubules could also be seen traveling in the opposite direction, into protrusions. We also show one example of a closer look at the leading edge. Membrane can be seen taken in from

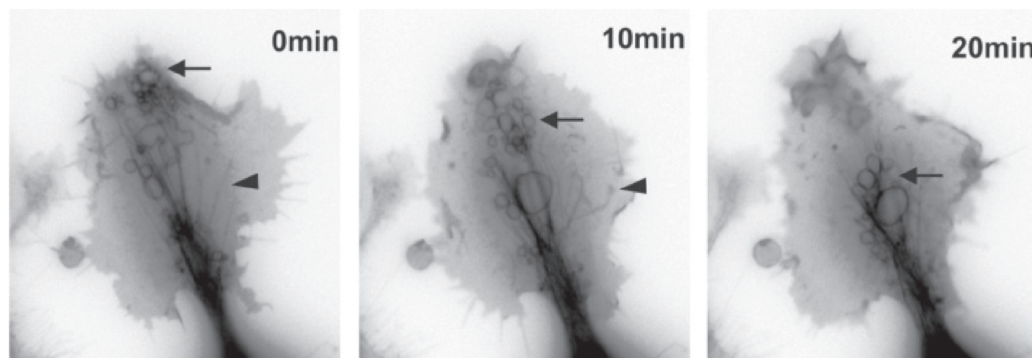



**Fig. 8. Formation of Rab8-specific macropinsomes at the leading edge of HT1080 fibrosarcoma cells.**

a ruffling area, traveling to a large vacuole from where membrane is then directed to the leading edge, promoting forward movement of lamellipodia. We conclude that the effect of Rab8 on cell polarity, i.e. formation of protrusions, is linked to membrane recycling at the leading edge.

We also studied the fate of Rab8 when stationary cells were incubated with the actin-depolymerizing agent cytochalasin D. Previous results of ours from fixed cells had shown that actin-depolymerization induces the formation of Rab8-specific tubules. Using live cell imaging, we could see that the tubules were formed in the cell periphery and moved towards the cell center where they accumulated as a tubular network. Membrane recycling in these cells is inhibited, leading to cell retraction. After cytochalasin D was washed off, new protrusions were formed, and the distribution of Rab8 was restored to the cell periphery (Peränen, unpublished).

#### *4.4.7 A model for the role of Rab8 in membrane traffic*

Our model for the membrane recycling system controlling the formation of polarized cell surface domains is illustrated in (Fig. 9). Rab8 controls a clathrin-independent membrane recycling route that operates in association with Arf6, is closely connected to actin dynamics, and dependent on microtubules. Protrusion formation is promoted by directing the transport of membrane components to specific cell surface domains. Our data clearly support the view that cell shape changes are not only controlled by actin and microtubules, but also by membrane turnover.

We have seen that endogenous Rab8 is associated with cell protrusions in free moving cells (publication II), but when cells form contacts Rab8 is relocalized to a diffuse perinuclear region (publication II). The Rab8-specific GEF, Rabin8, associates with actin-containing lamellipodia and ruffle-like structures at the plasma membrane, indicating that Rab8 is activated there (publication II).

In publication III we have looked at live cells and seen that macropinosomes and vesicles taken up at membrane ruffling areas fuse to form tubules and move inwards toward the center of the cell. Membrane is further transported back to the plasma membrane to form new cell protrusions. We have also seen that inhibiting this transport route by depletion of Rab8 or by expression of dominant negative mutants of Rab8, leads to cell-cell adhesion and abolishes protrusive activity.

Activated Rab8 produces a recycling compartment, consisting of vesicles and tubules. Arf6 and Rab8 colocalize on the same tubules and both induce the formation of protrusions (Peränen *et al*. 1996, Radhakrishna and Donaldson 1997, publication III). It is also known that Arf6-Q67L overexpression results in inhibition of ruffling, endocytosis as well as cell polarity (Brown *et al.* 2001, Santy 2002, Hashimoto *et al.* 2004b). In publication III we show that Arf6-Q67L inhibits the formation of Rab8-tubules and decreases the formation of Rab8-induced cell protrusions. An explanation for this could be that Arf6-GTP acts upstream of Rab8, by inhibiting membrane from reaching the Rab8-recycling compartment.

The Rab8-pathway is in publication III shown to contain several markers known to undergo internalization and recycling (β1-integrin, transferrin, transferrin receptor, and MHCI). It is further shown to contain several other markers known to participate in

membrane recycling or secretion (Arf6, JFC1, and Rab11). In accordance with several other reports our results indicate that the membrane of the Rab8-pathway, containing structural (adhesion receptors) and regulatory components (Arf6, Rho and Ras), is recycled back to the plasma membrane via a recycling compartment for the formation of new protrusions (Radhakrishna and Donaldson 1997, Ng *et al*. 1999, Furuhjelm and Peränen 2003, Deretic *et al*. 2004, Powelka *et al*. 2004, Schlunk *et al*. 2004).

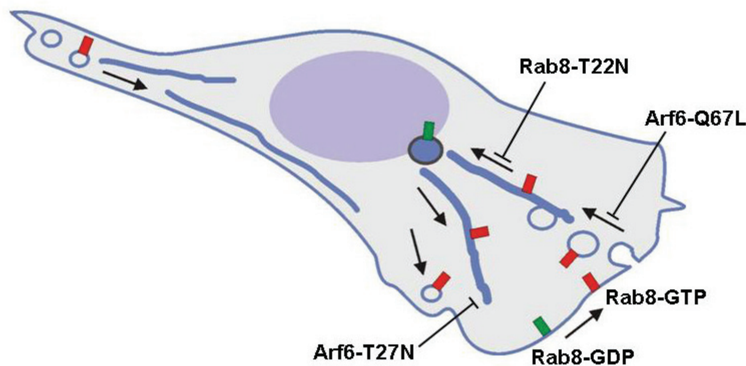



**Fig 9. Model of the Rab8/Arf6-recycling compartment.** Membrane is taken up by macropinocytosis at ruffling areas, a process regulated by Arf6-GTP. The membranes are delivered to the Rab8-recycling compartment after hydrolysis of GTP on Arf6. Activation of Rab8 produces a recycling compartment, consisting of vesicles and tubules, containing the recycled membrane components collected by Arf6. These membranes contain both structural (adhesion receptors) and regulatory (Arf6, Rho and Ras) components that are recycled back to the leading edge where they are needed to form new protrusions and a motile cell phenotype.

# 5. CONCLUSIONS AND FUTURE PERSPECTIVES

Out of the proteins known to date to interact with Rab8A (Fig. 10), three are presented in this thesis as novel Rab8-interacting proteins. In the two-hybrid screens I found many more putative Rab8-interacting proteins. I expect some of these to be of great interest when studied further, providing new insights as to the function of the Rab8 GTPase.

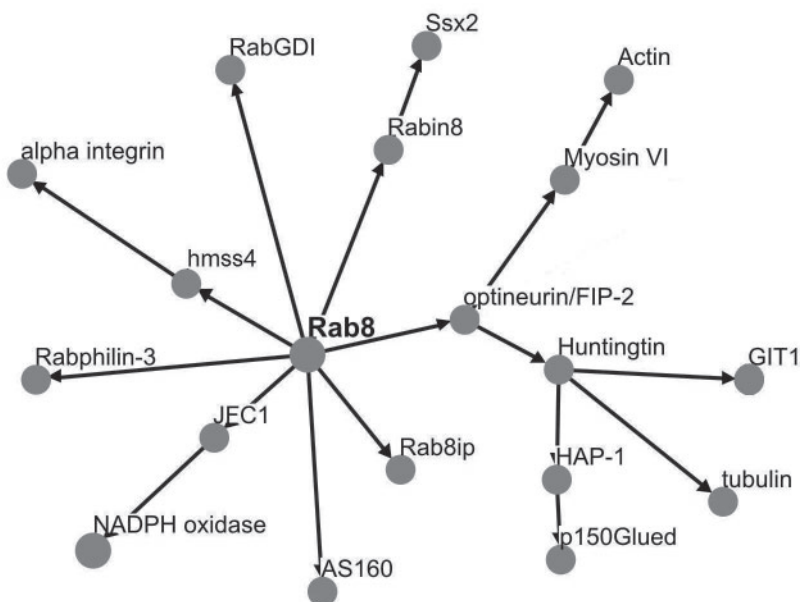



**Figure 10. The current Rab8A protein network.**

The results of this work supports a model in which Rab8 and Arf6 together constitute a recycling compartment for membranes participating in the formation of cell surface extensions. Furthermore, actin polymerization/depolymerization, controlled by RhoA, is vital to the function of Rab8 in controlling cell morphogenesis. Essential to unraveling the whole picture is finding the molecular links of Rab8 to both RhoA and Arf6. Experiments into how Arf6 and Rab8 cross-talk are currently underway.

Similarly, to unravel how Rab8 vesicles and tubules are transported along microtubules and actin filaments it is crucial to find molecular links between Rab8 and the cytoskeleton. Rabin8 could be one good candidate for a link with actin given that it localizes to actin-containing structures like lamellipodia and ruffle regions of the plasma membrane. We have not been able to show a direct interaction between Rabin8 and actin, however, a possible molecular link could be a myosin. Rab8 has been shown to co-localize with the expressed tail of myosin-Vc (Rodriguez and Cheney 2002). In addition Sahlender *et al.* (2005) have shown that optineurin/FIP-2 links myosin VI to the Golgi and that Rab8 recruits myosin VI onto Rab8-vesicles and tubules. Moreover, myosin VI has been shown to localize to ruffles and membrane traffic vesicles in a similar way as we have seen for Rab8 (Buss *et al*. 2002), making this particular myosin an interesting subject to study further.

What could be the molecular link to microtubules? It is known that Huntingtin binds tubulin (Hoffner *et al*. 2002), and HAP-1 links Huntingtin to dynactin, suggesting that Huntingtin could be part of a protein complex needed for the motility of membrane vesicles along microtubules (Engelender *et al.* 1997). Huntingtin has also been directly implicated in vesicular transport of neurotrophic factors along microtubules (Gauthiere *et al.* 2004). Could Huntingtin then provide a link to the transport of Rab8 vesicles along microtubules via the Rab8-interacting protein FIP-2/optineurin?

To further study the biogenesis of the Rab8-specific recycling compartment JFC1, the Rab8 interacting protein described in publication III, is interesting since its overexpression seems to increase association of Rab8 with tubular and vesicular structures after actin disruption, leading to a decrease in the number of tubules. Further studies are needed to sort out if this is due to JFC1 inhibiting the transformation of incoming vesicles into tubules or promoting the disassembly of existing tubules.

To date no difference in function has been detected between the two isoforms of Rab8 in mammals, Rab8A and Rab8b. They are highly homologous and differ basically only in the C-terminus. Both are found rather ubiquitously with the highest level of expression in rat for Rab8A being muscle lung and kidney, and for Rab8b, brain, spleen, and testis. It would be very interesting to try and find out how they differ and why. One of the polypeptides picked up in the two-hybrid screen did interact specifically with GTP-Rab8A but not GTP-Rab8b. Following this protein up might give some important clues.

We have shown that Rab8 has a clear role in the control of cell shape. How does it accomplish this? Our model proposes that Rab8-activity is needed to take in membrane from ruffle regions, no longer supporting adhesion, and recycling this material back to the plasma membrane for the formation of new protrusions. The membrane recycled in this way contains both structural (adhesion receptors) and regulatory components (Arf6, Rho and Ras). As well as being important for cell migration, strict control of cell polarization and protrusion formation would be especially important during embryogenesis and brain development. In neurite outgrowth long surface extensions are obviously needed. An important role for Rab8 in such specialized cell function could easily be envisaged. Depletion of Rab8 has in fact been shown to inhibit neurite outgrowth (Huber *et al.* 1995). In epithelial cells Rab8 could be required in the apical-basolateral polarity modulation as well as for the modulation of cell-cell contacts. In ADPKD (autosomal dominant kidney disease) cell loss of polarity is associated with redistribution of Rab8 (Charron *et al.* 2000). Moreover, Rab8b has been connected with adherens junction dynamics in the testis (Lau and Mruk 2003). Rapid renewal of membranes is needed for the development and maintenance of photoreceptors where depletion of Rab8 leads to cell death (Moritz *et al.* 2001). Rab8 could have a role in cancer cell invasion, bearing in mind that its expression has been shown to be up-regulated in breast cancer malignancies and their lymph node metastases (Hao *et al*. 2004). Finally, Rab8 could also be involved in processes needed for the completion of cell division, as has been shown for Arf6 (Schweitzer and D'Souza-Schorey 2002). Considering all of these findings, I anticipate that with further investigation Rab8 will be proven to play a significant role in many cellular processes where a rapid change in cell shape is needed.

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# REFERENCES


Abdul-Ghani M, Gougeon PY, Prossner DC, Da-Silva LF, and Ngsee JK. (2001). PRA isoforms are targeted to distinct membrane compartments. J. Biol. Chem. 276: 6225-6233.

Adams AE, Johnson DI, Longnecker RM, Sloat BF, and Pringle JR. (1990). CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast Saccharomyces cerevisiae. J. Cell Biol. 111: 131-142.

Allan BB, Moyer BD, and Balch WE. (2000). Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COP II vesicles for fusion. Science 289: 401-402.

Ananthakrishnan R and Ehrlicher A (2007). The forces behind movement. Int. J. Biol. Sci. 3: 303-317.

Ang AL, Fölsch H, Koivisto U-M, Pypaert M, and Mellman I. (2003). The Rab8 GTPase selectively regulates AP-1B-dependent basolateral transport in Madin-Darby canine kidney cells. J. Cell Biol. 163: 339-350.

Angst BD, Marcozzi C, and Magee AI. (2000). The cadherin superfamily: diversity in form and function. J. Cell Sci. 114: 629-641.

Armstrong J, Thompson N, Squire JH, Smith J, Hayes B, and Solari R. (1996). Identification of a novel member of the Rab8 family from the rat basophilic leukemia cell line, RBL.2H3. J. Cell Sci. 109: 1265-1274.

Arnaoutova I, Jackson CL, Al-Awar OS, Donaldson JG, and Loh JP. (2003). Recycling of Raft-associated prohormone sorting receptor carboxypeptidase E requires interaction with Arf6. Mol. Biol. Cell 14: 4448-4457.

Ayscough KR, Stryker J, Pokala N, Sanders M, Crews P, and Drubin DG. (1997). High rates of filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed by using the actin inhibitor latrunculin-A. J. Cell Biol. 137: 399-416.

Baas PW, Black MM, and Banker GA. (1989). Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. J. Cell Biol. 109: 3085-3094.

Badizadegan K, Wheeler HF, Fujinaga Y, and Lencer WI. (2004). Trafficking of cholera toxin-ganglioside GM1 complex into Golgi and induction of toxicity dependent on actin cytoskeleton. Am. J. Cell Physiol. 287: 1453-1463.

Bacallalo R, Antony C, Dotti C, Karsenti E, Stelzer EH, and Simons K. (1989). The subcellular organization of Madin-Darby canine kidney cells during the formation of a polarized epithelium. J. Cell Biol. 109: 2817-2832.

Ballestrem C, Hinz B, Imhof BA, and Wehrle-Haller B. (2001). Marching at the front and dragging behind: differential αvβ3-integrin turnover regulates focal adhesion behavior. J. Cell Biol. 155: 1319-1332.

Barbacid M. (1987). Ras genes. Ann. Rev. Biochem. 56, 779.

Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, Rexach MF, Ravazzola M, Amherdt M, and Schekman R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 77: 895-907.

Bar-Sagi D, and Feramisco JR. (1985). Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. Cell 42: 841-848.

Bergmann JE, Kupfer A, and Singer SJ. (1983). Membrane insertion at the leading edge of motile fibroblasts. Proc. Natl. Sci. USA 80: 1367-1371.

Bershadsky A, Chausovsky A, Becker E, Lyubimova A, and Geiger B. (1996). Involvement of microtubules in the control of adhesion-dependent signal transduction. Curr. Biol. 6: 1279-1289.

Bielli A, Thornqvist PO, Hendrick AG, Finn R, Fitzgerald K, and McCaffrey MW, (2001). The small GTPase Rab4A interacts with the central region of Cytoplasmic dynein light intermediate chain-1. Biochem. Biophys. Res. Commun. 281: 1141-1153.

Bilder D, Li M, and Perrimon N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science 289: 113-116.

Bilder D and Perrimon N. (2000). Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature 403: 676-680.

Birchmeier W, and Behrens J. (1994). Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim. Biophys. Acta 1198: 11-26.

Bischoff FR, Klebe C, Kretschmer J, Wittinghofer A, and Ponstingl H. (1994). RanGAP1 induces GTPase activity on nuclear Ras-related Ran. Proc. Natl. Acad. Sci. USA 91: 2587-2591.

Bischoff FR, and Ponstingl H. (1991). Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. Nature 354: 80-82.

Bishop AL, and Hall A. (2000). Rho GTPases and their effector proteins. Biochem. J. 348: 241-255.

Blagoveshchenskaya AD, Thomas L, Feliciangeli SF, Hung CH, and Thomas G. (2002). HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. Cell 111: 853-866.

Blangy A, Vignal E, Schmidt S, Debant A, Gauthier-Rouviere C, and Fort P. (2000). TrioGEF1 controls Rac- and Cdc42-dependent cell structures through the direct activation of rhoG. J. Cell Sci. 113: 729-739.

Bloom GS, and Goldstein LSB. (1998). Cruising along microtubule highways: how membranes move through the secretory pathway. J. Cell Biol. 140: 1277-1280.

Bock JB, and Scheller RH. (1997). Protein transport. A fusion of new ideas. Nature 387: 133-135.

Bourne HR, Sanders DA, and McCormick F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125-132.

Bourne HR, Sanders DA, and McCormick F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. Nature 349:117-127.

Bos J. (1989). Ras oncogenes in human cancer: a review. Cancer Res. 49, 4682.

Boshans RL, Szanto S, van Aelst L, D'Souza-Schorey C. (2000). ADP-ribosylation factor 6 regulates actin cytoskeleton remodeling in coordination with Rac1 and RhoA. Mol. Cell. Biol. 20: 3685-3694.

Bradke F and Dotti CG. (1999). The role of local actin instability in axon formation. Science 283: 1931- 1934.

Brakebusch C and Fässler R. (2003). The integrin-actin connection, an eternal love affair. EMBO J. 22: 2324-2333.

Breitfeld PP, McKinnon WC, Mostov KE. (1990). Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized MDCK cells. J. Cell Biol. 111: 2365-2373.

Bretscher MS, and Aquado-Velasco C. (1998). Membrane traffic during cell locomotion. Curr. Opin. Cell Biol. 10: 537-541.

Brondyk WH, McKiernan CJ, Fortner KA, Stabila P, Holz RW, and Macara IG. (1995). Interaction cloning of Rabin3, a novel protein that associates with the Ras-like GTPase Rab3A. Mol. Cell. Biol. 15: 1137-1143.

Brown AM, O'Sullivan AJ, and Gomperts BD. (1998). Induction of exocytosis from permeabilized mast cells by the guanosine triphosphatases Rac and Cdc42. Mol. Biol. Cell 9:1053-1063.

Brown FD, Rozelle AL, Yin HL, Balla T, and Donaldson JG. (2001). Phosphatidylinositol 4,5-biphosphate and Arf6-regulated membrane traffic. J. Cell Biol. 154: 1007-1017.

Brown MD, Cornejo BJ, Kuhn TB, and Bamburg JR. (2000). Cdc42 stimulates neurite outgrowth and formation of growth cone filopodia and lamellipodia. J. Neurobiol. 43: 352-364.

Brown R, Marshall CJ, Pennie SG, and Hall A. (1984). Mechanism of activation of an N-ras gene in the human fibrosarcoma cell line HT1080. EMBO J. 3: 1321-1326.

Bucci C, Chiariello M, Lattero D, Maiorano M, and Bruni CB. (1999). Interaction cloning and characterization of the cDNA encoding the human prenylated rab acceptor PRA1. Biochem. Biophys. Res. Commun. 258: 657-662.

Burgess DR (2005). Cytokinesis: new roles for myosin. Curr. Biol. 15: 310-311.

Burstein ES, and Macara IG. (1992). Characterization of a guanine nucleotide-releasing factor and a GTPase-activating protein that are specific for the ras-related protein p25rab3A. Proc. Natl. USA 89: 1154-1158.

Burton JL, Burns ME, Gatti E, Augustine GJ, and De Camilli P. (1994). Specific interactions of Mss4 with members of the Rab GTPase subfamily. EMBO J. 13: 5547-5558.

Buss F, Luszio JP, and Kendrick-Jones J. (2002). Myosin VI, an actin motor for membrane traffic and cell migration. Traffic 3: 851-858.

Cantalupo G, Alitano P, Bruni CB, and Bucci C. (2001). Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. EMBO J. 20: 683-693

Capon DJ, Seeburg PH, McGrath JP, Hayflick JS, Edman U, Levinson AD, and Goeddel DV. (1983). Activation of Ki-ras2 gene in human colon and lung carcinomas by two different point mutations. Nature 304: 507-513.

Carazo-Salas RE, Guarguaglini G, Gruss OJ, Segref A, Karsenti E, and Mattaj IW. (1999). Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. Nature 400: 178-181.

Carlier MF. (1998). Control of actin dynamics. Curr. Opin. Cell Biol. 10: 45-51.

Casey PJ and Seabra MC. (1998). Protein prenyltransferases. J. Biol. Chem. 271: 5389-5392.

Chabrillat ML, Wilhelm C, Wasmeier C, Sviderskaya EV, Louvard D, and Coudrier E. (2005). Rab8 regulates the actin-based movement of melanosomes. Mol. Biol. Cell 16: 1640-1650.

Chan AY, Bailly M, Zebda N, Segall JE, and Condeelis JS. (2000). Role of cofilin in epidermal growth factor-stimulated actin polymerization and lamellipod protrusion. J. Cell Biol. 148: 531-542.

Chant J and Stowers L. (1995). GTPase cascades choreographing cellular behavior: movement, morphogenesis, and more. Cell 81, 1-4.

Chant J. (1999). Cell polarity in yeast. Annu. Rev. Cell. Dev. Biol. 15: 365-391.

Charron AJ, Bacallao RL, and Wandinger-Ness A. (2000). ADPKD: a human disease altering Golgi function and basolateral exocytosis in renal epithelia. Traffic 8: 675-686.

Chavrier P, and Goud B. (1999). The role of ARF and Rab GTPases in membrane transport. Curr. Opin. Cell Biol. 11: 466-475.

Chen S, Liang MC, Chia JN, Ngseand JK, and Ting AE. (2001). Rab8b and its interacting partner TRIP8b are involved in regulated secretion in AtT20 cells. J. Biol. Chem. 276: 13209-13216.

Christoforidis S, McBride HM, Burgoyne RD, and Zerial M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. Nature 397: 621-625.

Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, Ivanov IE, and Philips MR. (1999). Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. Cell 98: 69-80.

Clark EA and Brugge JS. (1995). Integrins and signal transduction pathways: the road taken. Science 268: 233-239.

Cole NB, and Lippincott-Schwartz J. (1995). Organization of organelles and membrane traffic by microtubules. Curr. Opin. Cell Biol. 7: 55-64.

Colomer V, Engelender S, Sharp A, Duan K, Cooper J, Lanahan A, Lyford G, Worley P, and Ross CA. (1997). Huntingtin-associated protein 1 (HAP1) binds to a Trio-like polypeptide, with a rac1 guanine nucleotide exchange factor domain. Hum. Mol. Genet. 6: 1519-1525.

Cory GO and Ridley AJ. (2002). Cell motility: braking WAVEs. Nature 418: 732-733.

Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, and Gutkind JS. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 81: 1137-1146.

Craighead MW, Bowden S, Watson R, and Armstrong J. (1993). Function of the ypt2 gene in the exocytic pathway of Schizosaccharomyces pombe. Mol. Biol. Cell 4: 1069- 1076

Cunningham CC, Gorlin JB, Kwiatkowski DJ, Hartwig JH, Janmey PA, Byers HR, and Stossel TP. (1992). Actin-binding protein requirement for cortical stability and efficient locomotion. Science 255: 325-327.

Davies JA and Garrod DR. (1997). Molecular aspects of the epithelial phenotype. BioEssays 19: 699- 704.

de Curtis I. (2001). Cell migration: GAPs between membrane traffic and the cytoskeleton. EMBO Rep. 2 277-281.

De Marco N, Buono M, Troise F, and Diez-Roux G. (2006). Optineurin increases cell survival and translocates to the neucleus in a Rab8-dependent manner upon an apoptotic stimulus. J Biol. Chem. 281: 16147-16156.

Deretic D, Huber LA, Ransom N, Mancini M, Simons K, and Papermaster DS. (1995). Rab8 in retinal photoreceptors may participate in rhodopsin transport and in rod outer segment disk morphogenesis. J. Cell Sci. 108: 215-224.

Deretic D, Traverso V, Parkins N, Jackson F, Rodriguez de Turco EB, and Ransom N. (2004). Phosphoinositides, ezrin/moesin, and rac1 regulate fusion of rhodopsin transport carriers in retinal photoreceptors. Mol. Biol. Cell. 15: 359-370.

Diaz E, Schimmoler F, and Pfeffer SR. (1997). A novel Rab9 effector required for endosome-to TGN transport. J. Cell. Biol. 138: 283-290.

DiFiglia M, Sapp E, Chase K, Schwarz C, Meloni A, Young C, Martin E, Vonsattel JP, Carraway R, Reeves SA *et al.* (1995). Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. Neuron 14: 1075-1081.

Drubin D, Jones H, and Wertman K. (1993). Actin structure and function: roles in mitochondrial organization and morphogenesis in budding yeast and identification of the phalloidin-binding site. Mol. Biol. Cell 4: 1277-1294.

Donaldson JG. (2003). Multiple roles for Arf6: Sorting, structuring, and signaling at the plasma membrane. J. Biol. Chem. 278: 41573-41576.

Donladson JG and Honda A. (2005). Localization and function of Arf family GTPases. Biochem. Soc. Tans. 33: 639-642.

Douglass AD and Vale RD. (2005). Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. Cell 121: 937-950.

D'Souza-Schorey C, Li G, Colombo Mi, and Stahl PD. (1995). A regulatory role for ARF6 in receptor-mediated endocytosis. Science 267: 1175-1178.

D'Souza-Schorey C, van Donselaar E, Hsu VW, Yang C, Stahl PD, and Peters PJ. (1998). ARF6 targets recycling vesicles to the plasma membrane: insights from an ultrastructural investigation. J. Cell Biol. 140: 603-616.

El-Amraoui A, Schonn JS, Kussel-Andermann P, Blanchard S, Desnos C, Henry JP, Wolfrum U, Darchen F, and Petit C. (2002). MyRIP, a novel Rab effector, enables myosin VIIa recruitment to retinal melanosomes. EMBO Rep. 3: 463-470.

Elkind NB, Walch-Solimena C, and Novick PJ. (2000). The role of the COOH terminus of Sec2p in the transport of post-Golgi vesicles. J. Cell Biol. 149: 95-110.

Engelender S, Sharp A, Colomer V, Tokito M, Lanahan A, Worley P, Holzbaur E, and Ross C. (1997). Huntingtin-associated protein-1 (HAP1) interacts with the p150Glued subunit of dynactin. Hum. Mol. Genet. 6: 2205-2212.

Etienne-Manneville S and Hall A. (2002). Rho GTPases in cell biology. Nature 420: 629-635.

Etienne-Manneville S and Hall A. (2003). Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature 421: 753-756.

Euteneuer U and Schliwa M. (1992). Mechanism of centrosome positioning during the wound response in BSC-1 cells. J. Cell Biol. 116: 1157-1166.

Faber PW, Barnes GT, Srinidhi J, Chen J, Gusella JF, and MacDonald ME. (1998). Huntingtin interacts with a family of WW domain proteins. Hum. Mol. Genet. 7: 1463-1474.

Feramisco JR, Gross M, Kamata T, Rosenburg M, and Sweet RW. (1984). Microinjection of the oncogene form of the human H-Ras (T-24) protein results in rapid proliferation of quiescent cells. Cell 38: 109-117.

Finger FP, and Novick P. (1998). Spatial regulation of exocytosis: lessons from yeast. J. Cell Biol. 142: 609-612.

Finger FP, Hughes TE, and Novick P. (1998). Sec3p is a spatial landmark for polarized secretion in budding yeast. Cell 92: 559-571.

Franco M, Peters PJ, Boretto J, van Donselaar E, Neri A, D'Souza-Schorey C, and Chavier P. (1999). EFA6, a sec7 domain-containing exchange factor for ARF6, coordinates membrane recycling and actin cytoskeleton organization. EMBO J. 18: 1480-1491.

Frank SR, Hatfield JC, and Casanova JE. (1998). Remodeling of the actin cytoskeleton is coordinately regulated by protein kinase C and the ADP-ribosylation factor nucleotide exchange factor ARNO. Mol. Biol. Cell 9: 3133-3146.

Fuchs E, and Yang Y. (1999). Crossroads on cytoskeletal highways. Cell 98: 547-550.

Fukata Y, Kimura T, and Kaibuchi K. (2002). Axon specification in hippocampal neurons. Neurosci. Res. 43: 305-315.

Fukuda M. (2003). Slp4-a/granuphilin-a inhibits dense-core vesicle exocytosis through interaction with the GDP-bound form of Rab27a in PC12 cells. J. Biol. Chem. 278: 15390-15396.

Fukuda M, Kanno E, Saegusa C, Ogata Y, and Kuroda TS. (2002a). Slp-4a/Granuphilin-a regulates dense core vesicle exocytosis in PC12 cells. J. Biol. Chem. 42: 39673-39678.

Fukuda M, Kuroda TS, and Mikoshiba K. (2002b). Slac2-a/melanophilin, the missing link between Rab27 and myosin Va: implications of a tripartite protein complex for melanosome transport. J. Biol. Chem. 277: 12432-12436.

Fukuda M and Yamamoto A. (2005). Assay of the Rab-binding specificity of rabphilin and Noc2: target molecules for Rab27. Methods. Enzymol. 403: 469-481.

Furuhjelm J and Peränen J. (2003). The C-terminal end of R-Ras contains a focal adhesion targeting signal. J. Cell Sci. 116: 3729-3738.

Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H, Cordelieres FP, De Mey J, MacDonald ME, Lessmann V, Humbert S, and Saudou F. (2004). Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell 118: 127- 138.

Ge M, Cohen JS, Brown HA, and Freed JH. (2001). ADP ribosylation factor 6 binding to phosphatidylinositol 4,5-bisphosphate-containing vesicles creates defects in the bilayer structure: an electron spin resonance study. Biophys J. 81: 994-1005.

Gerges NZ, Backos DS, and Esteban JA. (2004). Local control of AMPA receptor trafficking at the post-synaptic terminal by a small GTPase of the Rab family. J. Biol. Chem. 279: 43870-43878.

Govek E-E, Newey SE, and Van Aelst L. (2005). The role of the Rho GTPases in neuronal development. Genes and development 19: 1-49.

Govindan B, Bowser R, and Novick P. (1995). The role of Myo2, a yeast class V myosin, in vesicular transport. J. Cell. Biol. 128: 1055-1068.

Gumbiner BM. (1993). Proteins associated with the cytoplasmic surface of adhesion molecules. Neuron 11: 551-564.

Gumbiner BM. (1996). Cell adhesion: the molecular basis of tissue architechture and morphogenesis. Cell 84: 345-357.

Gundersen GG, and Bulinski JC. (1988). Selective stabilization of microtubules oriented towards the direction of cell migration. Proc. Natl. Acad. Sci. USA 85: 5946-5950.

Gundersen GG, Gomes ER, and Wen Y. (2004). Cortical control of microtubule stability and polarization. Curr. Opin. Cell Biol. 16: 1-7.

Guo W, Roth D, Walch-Solimena C, Novick P. (1999). The exocyst is an effector of Sec4p, targeting secretory vesicles to sites of exocytosis. EMBO J. 18: 1071-1080.

Guzik BW and Goldstein LSB. (2004). Microtubule-dependent transport in neurons: steps towards an understanding of regulation, function and dysfunction. Curr. Opin. Cell Biol. 16: 1- 8.

Haas AK, Fuchs E, Kopajtich R, and Barr FA. (2005). A GTPase-activating protein controls Rab5 function in endocytic trafficking. Nat. Cell Biol. 7: 887-893.

Hales CM, Griner R, Hobdy-Henderson KC, Dorn MC, Hardy D, Kumar R, Navarre J, Chan EK, Lapierre LA, and Goldenring JR. (2001). Identification and characterization of a family of Rab11-interacting proteins. J. Biol. Chem. 276: 39067-39075.

Hall A. (2000). GTPases. Oxford Univ. Press, Oxford.

Hao X, Sun B, Hu L, Lahdesmaki H, Dunmire V, Feng Y, Zhang SW, Wang H, Wu C, Wang H, Fuller GN, Symmans WF, Shmulevich I, and Zhang W. (2004). Differential gene and protein expression in primary breast malignancies and their lymph node metastases as revealed by combined cDNA microarray and tissue microarray analysis. Cancer 100: 1110-1122.

Hart MJ, Eva A, Zangrilli D, Aaronson SA, Evans T, Cerione RA, and Zheng Y. (1994). Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogenes product. J. Biol. Chem. 269: 62-65.

Hashimoto S, Hashimoto A, Yamada A, Kojima C, Yamamoto H, Tsutsumi T, Higashi M, Mizoguchi A, Yagi R, and Sabe H. (2004)a. A novel mode of action of an ArfGAP, AMAP2/PAG3/Papα, in Arf6 function. J. Biol. Cem. 279: 37677-37684.

Hashimoto S, Onodera Y, Hashimoto A, Tanaka M, Hamaguchi M, Yamada A, and Sabe H. (2004)b. Requirement for Arf6 in breast cancer invasive activities. Proc. Natl. Sci. USA 101: 6647-6652.

Hickson GR, Matheson J, Riggs B, Maier VH, Fielding AB, Prekeris R, Sullivan W, Barr FA, and Goud GW. (2003). Arfophilins are dual Arf/Rab11 binding proteins that regulate recycling endosome distribution and are related to Drosophila nuclear fallout. Mol. Biol. Cell 14: 2908-2920.

Hill CS, Wynne J, and Treisman R. (1995). The Rho-family GTPases RhoA, Rac1, and Cdc42Hs regulate transcriptional activation by SRF. Cell 81: 1159-1170.

Hoffner G, Kahlem P, and Djian P. (2002). Perinuclear localization of huntingtin as a consequence of its binding to microtubules through an interaction with β-tubulin: relevance to Huntington's disease. J. Cell Sci. 115: 941-948.

Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, Kawamoto K, Nakayama K, Morris AJ, Frohman MA, and Kanaho Y. (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell 99: 521-532.

Hopkins CR, Gibson A, Shipman M, Strickland DK, and Trowbridge IS. (1994). In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J. Cell Biol. 125: 1265-1274.

Horiuchi H, Lippe R, McBride HM, Rubino M, Woodman P, Stenmark H, Rybin V, Wilm M, Ashman K, Mann M, and Zerial M. (1997). A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. Cell 90: 1149-1159.

Horton AC and Ehlers MD. (2003). Neuronal polarity and trafficking. Neuron 40: 277- 295.

Hsu SC, Ting AE, Hazuka CD, Davanger S, Kenny JW, Kee Y, and Scheller RH. (1996). The mammalian brain rsec6/8 complex. Neuron 17: 1209-1219.

Huber LA, deHoop MJ, Dupree P, Zerial M, and Dotti C. (1993b). Protein transport to the dendritic plasma membrane of cultured neurons is regulated by Rab8p. J. Cell Biol. 123: 47-55.

Huber LA, Dupree P, and Dotti CG. (1995). A deficiency of the small GTPase rab8 inhibits membrane traffic in developing neurons. Mol. Cell. Biol. 15: 918-924.

Huber LA, Pimplikar S, Parton RG, Virta H, Zerial M, and Simons K. (1993a). Rab8A, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. J. Cell Biol. 123: 35-45.

Hunker CM, Galvis A, Kruk I, Gambini H, Veisaga ML, and Barbieri MA. (2006). Rab5-activating protein 6, a novel endosomal protein with a role in endocytosis. Biochem. Biophys. Res. Commun. 340: 967-975.

Huttenlocher A, Sandborg PR, and Horowitz AF. (1995). Adhesion in cell migration. Curr. Opin. Cell Biol. 7: 697-706.

Hynes RO. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69: 11-25.

Hynes RO (2002). Integrins: bidirectional, allosteric signaling machines. Cell 110: 673-687.

Imamura H, Takaishi K, Nakano K, Kodama A, Oishi H, Shiozaki H, Monden M, Sasaki T, and Takai Y. (1998). Rho and Rab small G proteins coordinately reorganize stress fibers and focal adhesions in MDCK cells. Mol. Biol. Cell 9: 2561-2575.

Iwasaki K and Toyonaga R. (2000). The Rab3 GDP/GTP exchange factor homolog AEX-3 has a dual function in synaptic transmission. EMBO J. 19: 4806-4816.

Izaurralde E, Kutay U, von Kobbe C, Mattaj IW, and Gorlich D. (1997). The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. EMBO J. 16: 6535-6547.

Jackson CL, and Casanova JE. (2000). Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. Trends. Cell Biol. 10: 60-67.

Johnson DI, and Pringle JR. (1990). Molecular characterization of CDC42, a Saccharomyces cerevisiae gene involved in the development of cell polarity. J. Cell Biol. 111: 143-152.

Johnson JJ, Ellis BA, Noack D, Seabra MC, and Catz SD. (2005a). The Rab27a-binding protein, JFC1, regulates androgen-dependent secretion of prostate-specific antigen and prostatic-specific acid phosphatase. Biochem. J. 391: 699-710.

Johnson JJ, Pacquelet S, Lane WS, Eam B, and Catz SD. (2005b). Akt regulates the subcellular localization of the Rab27a-binding protein JFC1 by phosphorylation. Traffic 6: 667-681.

Johnston G, Pendergast J, and Singer R. (1991). The Saccharomyces cerevisiae MYO2 gene encodes an essential myosin for vectorial transport of vesicles. J. Cell Biol. 113: 539-551.

Jou TS and Nelson WJ. (1998). Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. J. Cell Biol. 142: 85- 100

Kahana JA, and Cleveland DW. (1999). Beyond nuclear transport. Ran-GTP as a determinant of spindle assembly. J. Cell Biol. 146: 1205-1210.

Kaksonen M, Toret CG, and Drubin DG. (2006). Harnessing actin dynamics for clathrin-mediated endocytosis. Nat Rev Mol Cell Biol. 7: 404-414.

Kamei T, Matozaki T, Sakisaka T, Kodama A, Yokoyama S, Peng YF, Nakano K, Takaishi K, and Takai Y. (1999). Coendocytosis of cadherin and c-Met coupled to disruption of cell-cell adhesion in MDCK cells- regulation by Rho, Rac and Rab small G proteins. Oncogene 18: 6776-6784.

Katz P, Whalen G, and Kehrl JH. (1994). Differential expression of a novel protein kinase in human B lymphocytes. Prefential localization in the germinal center. J. Virol. Chem. 269: 16802- 16809.

Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, Gilbert C, Coffer P, Downward J, and Evan G. (1997). Supression of c-Myc-induced apoptosis by Ras signaling through PI(3)K and PKB. Nature 385: 544-548.

Keller P, Toomre D, Diaz E, White J, and Simons K. (2001). Multicolour imaging of post-Golgi sorting and trafficking in live cells. Nat. Cell Biol. 3: 140-149.

Kemler R, Ozawa M, and Ringwald M. (1989). Calcium-dependent cell adhesion molecules. Curr. Opin. Cell Biol. 1: 892-897.

Kirkham M, Fujita A, Chadda R, Noxon SJ, Kurzchalia TV, Sharma DK, Pagano RE, Hancock JF, Mayor S, and Parton RG. (2005). Ultrastructural identification of uncoated cavelolin-independent early endocytic vehicles. J. Cell Biol. 168: 465-476.

Knust E, Tepass U, and Wodarz A. (1993). Crumbs and stardust, two genes of Drosophila required for the development of epithelial cell polarity. Dev. Suppl. 261-268.

Kodama A, Lechler T and Fuchs E. (2004). Coordinating cytoskeletal tracks to polarize cellular movements. J. Cell Biol. 2: 203-207.

Komuro R, Sasaki T, Takaishi K, Orita S, and Takai Y. (1996). Involvement of Rho and Rac small G proteins and Rho GDI in Ca2+-dependent exocytosis from PC12 cells. Genes Cells 1: 943-951.

Kozma R, Ahmed S, Best A, and Lim L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol. Cell. Biol. 15: 1942-1952.

Kron SJ, Drubin DG, Botstein D, and Spudich JA. (1992). Yeast actin filaments display ATP-dependent sliding movement over surfaces coated with rabbit muscle myosin. Proc. Natl. Acad. Sci. USA 89: 4466-4470.

Kroschewski R, Hall A, and Mellman I. (1999). Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. Nat. Cell Biol. 1: 8-13.

Kuge O, Dascher C, Orci L, Rowe T, Amherdt M, Plutner H, Ravazzola M, Tanigawa G, Rothman JE, and Balch WE. (1994). Sar1 promotes vesicle budding from the endoplasmic reticulum but not Golgi compartments. J. Cell Biol. 125: 51-65.

Lamaze C, Chuang TH, Terlecky LJ, Bokoch GM, and Schmid SL. (1996). Regulation of receptor-mediated endocytosis by Rho and Rac. Nature 382: 177-179.

Lanier LM, and Gertler FB. (2000). Actin cytoskeleton: thinking globally, actin' locally. Curr. Biol. 10: R655-R657.

Lanzetti L, Palamidessi, Areces L, Scita G, and Di Fiore PP. (2004). Rab5 is a signaling GTPase involved in actin remodeling by receptor tyrosine kinases. Nature 429: 309-314.

Lanzetti L, Rybin V, Malabarba MG, Christoforidis S, Scita G, Zerial M, and Di Fiore PP. (2000). The Eps8 protein coordinates EGF receptor signaling through Rac and trafficking through Rab5. Nature 408: 374-377.

Lapierre LA, Kumar R, Hales CM, Navarre J, Bhartur SG, Burnette JO, Provance DW Jr., Mercer JA, Bahler M, and Goldenring JR. (2001). Myosin Vb is associated with plasma membrane recycling systems. Mol. Biol. Cell 12: 1843- 1857.

Lau AS, and Mruk DD. (2003). Rab8b GTPase and junction dynamics in the testis. Endocrinology 144: 1546-1563.

Lauffenberger DA, and Horwitz AF. (1996). Cell migration: a physically integrated molecular process. Cell 84: 359-369.

Laukaitis CM, Webb DJ, Donais K, and Horowitz AF. (2001). Differential dynamics of α5 integrin, paxillin, and α-actinin during formation and disassembly of adhesions in migrating cells. J. Cell Biol. 153: 1427-1440.

Lazar T, Gotte M, and Gallwitz D. (1997). Vesicular transport: how many Ypt/Rab-GTPases makes a eukaryotic cell? Trends Biochem. Sci. 22: 468-472.

Lee S, Fan S, Makarova O, Straight S, and Margolis B. (2002). A novel and conserved protein-protein interaction domain of mammalian Lin-2/CASK binds and recruits SAP97 to the lateral surface of epithelia. Mol. Cell. Biol. 22: 1778-1791.

Li L, Omata W, Kojima I, and Shibata H. (2001). Direct interaction of Rab4 with syntaxin 4. J. Biol. Chem. 276: 5265-5273.

Li S-H, Li H, Torre E, and Li X-J. (2000). Expression of Huntingtin-associated protein-1 in neuronal cells implicates a role in neuritic growth. Mol. Cell. Neurosci. 16: 168-183.

Li Y, Kang J, and Horowitz MS. (1998). Interaction of an adenovirus E3 14.7-kilodalton protein with a novel tumor necrosis factor alpha-inducible cellular protein containing leucine zipper domains. Mol. Cell. Biol. 18: 1601-1610.

Liang JO and Kornfeld S. (1997). Comparative activity of ADP-ribosylation factor family members in the early steps of coated vesicle formation on rat liver Golgi membranes. J. Biol. Chem. 272: 4141-4148.

Liang JO, Sung TC, Morris AJ, Frohman MA, and Kornfeld S. (1997). Different domains of mammalian ADP-ribosylation factor 1 mediate interaction with selected target proteins. J. Biol. Chem. 272: 33001-3308.

Liao G, Nagasaki T, and Gundersen GG. (1995). Low concentrations of nocodazole interfere with fibroblast locomotion without significantly affecting microtubule level: implications for the role of dynamic microtubules in cell locomotion. J. Cell Sci. 108: 3473- 3483.

Lillie S, and Brown S. (1994). Immunofluorescence localization of the unconventional myosin, Myo2p, and the putative kinesin-related protein, Smy1p to the same regions of polarized growth in *Saccharomyces cerevisiae*. J. Cell Biol. 125: 825-842.

Lindsay AJ, Hendrick AG, Cantalupo G, Senic-Matuglia F, Goud B, Bucci C, and McCaffrey MW. (2002). Rab coupling protein (RCP), a novel Rab4 and Rab11 effector protein. J. Biol. Chem. 277: 12190-12199.

Lindsay and McCaffrey (2002). Rab11-FIP2 functions in transferrin recycling and associates with endosomal membranes via its COOH-terminal domain. Biochem. Biophys. Res. Commun. 277: 27193-27199.

Liu K and Li G. (1998). Catalytic domain of the p120 Ras GAP binds to Rab5 and stimulates its GTPase activity. J. Biol. Chem. 273: 10087-10090.

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, and Darnell J. (1999). Molecular cell biology. Fourth edition. W.H. Freeman and Company, New York.

Luo HR, Saiaridi A, Nagata E, Ye K, Yu H, Jung TS, Luo X, Jain S, Sawa A, and Snyder SH. (2001). GRAB. A physiological guanine nucleotide exchange factor for Rab3A, which interacts with inositol hexakisphosphate kinase. Neuron 31: 439-451.

Luthi-Carter R, Strand A, Peters N, Solano S, Hollingsworth Z, Menon A, Frey AS, Spektor BS, Penney EB, Schilling G, Ross CA, Borchelt DR, Tapscott SJ, Young AB, Cha JH, and Olson JM. (2000). Decreased expression of striatal signaling genes in a mouse model of Huntington's disease. Hum. Mol. Genet. 9: 1259-1271.

Luton F, Klein S, Chauvin JP, Le Bivic A, Bourgoine S, Franco M, and Chardin P. (2004). EFA6, exchange factor for ARF6, regulates the actin cytoskeleton and associated tight junction in respons to E-cadherin engagement. Mol. Biol. Cell 15:1134-1145.

Madden K, and Snyder M. (1998). Cell polarity and morphogenesis in budding yeast. Annu. Rev. Microbiol. 52: 687-744.

Magee AI, Newman CM, Giannakouros T, Hancock JF, Fawell E, and Armstrong J. (1992). Lipid modifications and function of the ras superfamily of proteins. Biochem. Soc. Trans. 20: 497-499.

Mammoto A, Ohtsuka T, Hotta I, Sasaki T, and Takai Y. (1999). Rab11 BP/Rabphilin-11, a downstream target of rab11 small G protein. J. Biol. Cem. 274: 25517-25524.

Massol RH, Larsen JE, Fujinga Y, Lencer WI, and Kirchhausen T. (2004). Cholera toxin toxicity does not require functional Arf6- and dynamin-dependent endocytic pathways. Mol. Biol. Cell 15: 3631-3641.

Matter K, Bucher K, and Hauri HP. (1990). Microtubule perturbation retards both the direct and the indirect apical pathway but does not affect sorting of plasma membrane proteins in intestinal epithelial cells (Caco-2). EMBO J. 9: 3163-3170.

McAdara-Berkowitz JK, Catz SD, Johnson JL, Ruedi JM, Thon V, and Babior BM. (2001). JFC1, a novel tandem repeat C2 domain-containing protein associated with the leukocyte NADPH oxidase. J. Biol. Chem. 276: 18855-18862.

Medina E, Lemmers C, Lane-Guermonprez L, and Le Bivic A. (2002). Role of the Crumbs complex in the regulation of junction formation in Drosophila and mammalian epithelial cells. Biol. Cell. 94: 305-313.

Melendez AJ, Harnett MM, and Allen JM. (2001). Crosstalk between ARF6 and protein kinase Calpha in Fc(gamma)RI-mediated activation of phospholipase D1. Curr. Biol. 11: 869-874.

Mesa R, Magadan J, Barbieri A, Lopez C, Stahl PD, and Mayorga LS. (2005). Overexpression of Rab22a hampers the transport between endosomes and the Golgi apparatus. Exp. Cell Res. 304: 339-353.

Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peränen J, Lane WS, and Lienhard GE. (2005). AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem. J. 391: 87-93.

Millan J, Qaidi M, and Alonso MA. (2001). Segregation of co-stimulatory components into T cell surface lipid rafts. Eur. J. Immunol. 31: 467-473.

Miura Y, Kikuchi A, Musha T, Kuroda S, Yaku H, Sasaki T, and Takai Y. (1993). Regulation of morphology by rho p21 and its inhibitory GDP/GTP exchange protein (rhoGDI) in Swiss 3T3 cells. J. Biol. Chem. 268: 510-515.

Miyoshi J and Takai Y. (2005). Molecular perspective on tight-junction assembly and epithelial polarity. Adv. Drug Del. Rev. 57: 815-855.

Mizuno K, Kitamura A, and Sasaki T. (2003). Rabring7, a novel Rab7 target protein with a RING finger motif. Mol. Biol. Cell 14: 3741-3752.

Monier S, Jollivet F, Janoueix-Lerosey I, Johannes L, and Goud B. (2002). Characterization of novel Rab6-interacting proteins involved in endosome-to-TGN transport. Traffic 3: 289-297.

Moon SY and Zheng Y. (2003). Rho GTPase-activating proteins in cell regulation. Trends Cell Biol. 13: 13-22.

Moore MS. (1998). Ran and nuclear transport. J. Biol. Chem. 273: 22857-22860.

Moore MS and Blobel G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature 365: 661-663.

Moritz OL, Tam BM, Hurd LL, Peränen J, Deretic D, and Papermaster DS. (2001). Mutant rab8 impairs docking and fusion of rhodopsin-bearing post-Golgi membranes and causes cell death of transgenic Xenopus rods. Mol. Biol. Cell 12: 2341-2351.

Moss J, and Vaughan M. (1995). Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. J. Biol. Chem. 270: 12327-12330.

Mostov KE. (1995). Regulation of protein traffic in polarized epithelial cells. Histology & Histopathology 10: 423-431.

Muto A, Arai KI, and Watanabe S. (2006). Rab11-FIP4 is predominantly expressed in neural tissues and involved in proliferation as well as differentiation during zebrafish retinal development. Dev. Biol. 292: 90-102.

Nabi IR. (1999). The polarization of the motile cell. J. Cell Sci. 112: 1803-1811.

Nakada C, Ritchie K, Oba Y, Nakamura M, Hotta Y, Iino R, Kasai RS, Yamaguchi K, Fujiwara T, and Kusumi A. (2003). Accumulation of anchored proteins forms membrane diffusion barriers during neuronal polarization. Nat. Cell Biol. 5: 626-632.

Nakano A and Muramatsu M. (1989). A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. J. Cell. Biol. 109: 2677-2691.

Nefsky B, and Bretcher A. (1992). Yeast actin is relatively well behaved. Eur. J. Biochem. 206: 949-955.

Ng T, Shima D, Squire A, Bastiaens PI, Gschmeissner S, Humphries MJ, and Parker PJ. (1999). PKCα regulates β1 integrin-dependent cell motility through association and control of integrin traffic. EMBO J. 18: 3909-3923.

Nichols BJ, Ungermann P, Pelham HR, Wickner WT, and Haas A. (1997). Homotypic vacuolar fusion mediated by t- and v-SNAREs. Nature 387:199-202.

Nie Z, Hirsch DS, and Randazzo PA. (2003). Arf and its many interactors. Curr. Opin. Cell Biol. 15: 396-404.

Nishikawa S and Nakano A. (1991). The GTP-binding Sar1 protein is localized to the early compartment of the yeast secretory pathway. Biochim. Biophys. Acta. 1093: 135-143.

Nishimura N, Nakamura H, Takai Y, and Sano K. (1994). Molecular cloning and characterization of two rab GDI species from rat brain: brain-specific and ubiquitous types. J. Biol. Chem. 269: 14191-14198.

Nobes CD, and Hall A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81: 53-62.

Nobes CD, and Hall A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. 144: 1235-1244.

Novick P, Field C, and Schekman R. (1980). Identification of 23 complementation groups required for posttranslational events in the yeast secretory pathway. Cell 21: 205-215.

Novick P, and Zerial M. (1997). The diversity of Rab proteins in vesicle transport. Curr. Opin. Cell Biol. 9: 496-504.

Nuoffer C, and Balch WE. (1994). GTPases: multifunctional molecular switches regulating vesicular traffic. Annu. Rev. Biochem. 63: 949-990.

Nuoffer C, Wu SK, Dascher C, and Balch WE. (1997). Mss4 does not function as an exchange factor for Rab in endoplasmic reticulum to Golgi transport. Mol. Cell. Biol. 8: 1305-1315.

O'Connell KF, Maxwell KN, and White JG. (2000). The spd-2 gene is required for polarization of the anteroposterior axis and formation of the sperm asters in the Caenorhabditis elegans zygote. Dev. Biol. 222: 55-70.

Ohno M, Fornerod M, and Mattaj IW. (1998). Nucleocytoplasmic transport: the last 200 nanometers. Cell 92: 327-336.

Olkkonen VM, and Stenmark H. (1997). Role of Rab GTPases in membrane traffic. Int. Rev. Cytol. 176: 1-85.

Palacios F, Price L, Schweitzer J, Collard JG, and D'Souza-Schorey C. (2001). An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. EMBO J. 20: 4973-4986.

Palacios F, Schweitzer JK, Boshans RL, and D'Souza-Schorey C. (2002). ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. Nat. Cell Biol. 4: 929-936.

Perona R, Montaner S, Saniger L, Sanchez-Perez I, Bravo R, and Lacal JC. (1997). Activation of the nuclear factor-kappaB by Rho, Cdc42, and Rac-1 proteins. Genes Dev. 11: 463-475.

Peränen J, Auvinen P, Virta H, Wepf R, and Simons K. (1996). Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. J. Cell Biol. 135: 153-167.

Peränen J and Furuhjelm J. (2001). Expression, purification, and properties of Rab8 function in actin cortical skeleton organization and polarized transport. Methods. Enzymol. 329: 188-196.

Peden AA, Schonteich E, Chun J, Junutula JR, Scheller RH, and Prekeris R. (2004). The RCP-Rab11 complex regulates endocytic protein sorting. Mol. Biol. Cell 15: 3530-3541.

Peters PJ, Hsu VW, Ooi CE, Finazzi D, Teal SB, Oorschot V, Donaldson JG, and Klausner RD. (1995). Overexpression of wild-type and mutant ARF1 and ARF6: distinct perturbations of nonoverlapping membrane compartments. J. Cell Biol. 128: 1003-1017.

Pfeffer S. (2005). A model for Rab GTPase localization. Biochem. Soc. Trans. 33: 627-630.

Pierini LM, Lawson MA, Eddy RJ, Hendey B, and Maxfield FR. (2000). Oriented endocytic recycling of alpha5beta1 in motile neutrophils. Blood 95: 2471-2480.

Pollard TD and Borisy GG. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell 112: 453-465.

Powelka AM, Sun J, Li J, Gao M, Shaw LM, Sonnenberg A, and Hsu VW. (2004). Stimulation-dependent recycling of integrinβ1 regulated by ARF6 and Rab11. Traffic 5: 20-36.

Powner DJ, Hodgkin MN, and Wakelam MJ. (2002). Antigen-stimulated activation of phospholipase D1b by Rac1, ARF6, and PKCalpha in RBL-2H3 cells. Mol. Biol. Cell 13: 1252-1262.

Praefcke GJK, and McMahon HT. (2004). The dynamin superfamily: universal membrane tabulation and fission molecules? Nature 5: 133-147.

Prekeris R, Davies JM, and Scheller RH. (2001). Identification of a novel Rab11/25 binding domain present in Eferin and Rip proteins. J. Biol. Chem. 276: 38966-38970.

Prekeris R, Klumperman J, and Scheller RH. (2000). A Rab11/Rip11 protein complex regulates apical membrane trafficking via recycling endosomes. Mol. Cell 6: 1437-1448.

Pruyne DW, Schott DH, Bretscher A. (1998). Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. J. Cell Biol. 143: 1931-1945.

Qiu RG, Abo A, and Steven Martin G. (2000). A human homolog of the C. elegans polarity determinant Par-6 links Rac and Cdc42 to PKCzeta signaling and cell transformation. Curr Biol. 10: 697-707.

Qualmann B and Kessels MM. (2002). Endocytosis and the cytoskeleton. Int. Rev. Cytol. 220: 93-144.

Rahl PB, Chen CZ, and Collins RN. (2005). Elp1, the yeast homolog of the FD disease syndrome Protein, Negatively Regulates Exocytosis Independently of Transcriptional Elongation. Mol. Cell 17:841-853.

Radhakrishna H, Al-Awar O, Khachikian Z, and Donaldson JG. (1999). ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. J. Cell Sci. 112: 855-866.

Radhakrishna H and Donaldson JG. (1997). ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. J. Cell Biol. 139: 49-61.

Raftopoulou M and Hall A. (2004). Cell migration: Rho GTPases lead the way. Dev. Biol. 265: 23-32.

Rameh LE, Arvidsson A, Carraway KLR, Couvillon AD, Rathburn G, Crompton A, Van Renterghem B, Czech MP, Ravichandran KS, Burakoff SJ, et al (1997). A comparative analysis of the phosphoinositides binding specificity of plextrin homology domains. J. Biol. Chem. 272: 22059-22066.

Randazzo PA and Hirsch DS. (2004). Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodelling. Cell. Signal. 16: 401-413.

Ren M, Zeng J, De Lemos-Chiarandini C, Rosenfeld M, Adesnik M, and Sabatini DD. (1996). In its active form, the GTP-binding protein rab8 interacts with a stress-activated protein kinase. Proc. Natl. Acad. Sci. USA 93: 5151-5155.

Rezaie T, Child A, Hitchings R, Brice G, Miller L, Coca-Prados M, Heon E, Krupin T, Ritch R, Kreutzer D, Crick RP, and Sarfarazi M. (2002). Adult-onset primary open-angle glaucoma caused by mutations in optineurin. Science 295: 1077-1079.

Ridley AJ, and Hall A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70: 389-399.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, and Hall A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70: 401-410.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, and Horowitz AR. (2003). Cell migration: integrating signals from front to back. Science 302: 1704-1709.

Rigamonti D, Bauer J, De-Fraja C, Conti L, Sipione S, Sciorati C, Clementi E, Hackam A, Hayden MR, Li Y, Cooper JK, Ross CA, Govoni S, Vincenz C, and Cattaneo E. (2000). Wild-type Huntingtin protects from apoptosis upstream of caspase-3. J. Neurosci. 20: 3705-3713.

Rinnerthaler G, Geiger B, and Small JV. (1988). Contact formation during fibroblast locomotion: involvement of membrane ruffles and microtubules. J. Cell Biol. 106: 747- 760.

Rodriguez J, and Paterson B. (1990). Yeast myosin heavy chain mutant: maintenance of the cell type specific budding pattern and the normal deposition of chitin and cell wall components requires an intact heavy chain gene. Cell Motil. Cytoskelet. 17: 301-308.

Rodriguez OC, and Cheney RE. (2002). Human myosin-Vc is a novel class V myosin expressed in epithelial cells. J. Cell Sci. 115: 991-1004.

Rodriguez-Gabin AG, Cammer M, Amlazan G, Charron M, and Larocca JN. (2001). Role of rRab22b, an oligodendrocyte protein, in regulation of transport of vesicles from trans Golgi to endocytic compartments. J. Neurosci. Res. 66: 1149-1160.

Rojas R and Apodaca G. (2002). Immunoglobulin transport across polarized epithelial cells. Nat. Rev. Mol. Cell Biol. 3: 944-955.

Rosa JL, Casaroli-Marano RP, Buckler AJ, Vilaro S, and Barbacid M. (1996). p619, a giant protein related to the chromosome condensation regulator RCC1, stimulates guanine nucleotide exchange on ARF1 and Rab proteins. EMBO J. 15: 4262- 4273.

Rosen E and Goldberg I. (1989). Protein factors which regulate cell motility. In Vitro Cell. Dev. Biol. 25: 1079-1087.

Roth MG. (2000). Arf. In GTPases, edited by Hall A. Oxford UK: Oxford Univ. Press p176-197.

Rothman JE. (1996). The protein machinery of vesicle budding and fusion. Protein Sci. 5: 185-194.

Rush MG, Drivas G, and D'Eustachio P. (1996). The small nuclear GTPase Ran: how much does it run? Bioessays 18: 103-112.

Sahlender DA, Roberts RC, Arden SD, Spudich G, Taylor MJ, Luzio JP, Kendrick-Jones J, and Buss F. (2005). Optineurin links myosin VI to the Golgi complex and is involved in Golgi organization and exocytosis. J. Cell Biol. 169: 285- 295.

Salminen A, and Novick PJ. (1987). A ras-like protein is required for a post-Golgi event in yeast secretion. Cell 49: 527-538.

Santy LC. (2002). Characterization of fast recycling ADP-ribosylation factor 6 mutant. J. Biol. Chem. 277: 40185-40188.

Santy LC and Casanova JE. (2001). Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. J. Cell Biol. 154: 599-610.

Santy LC and Casanova JE. (2002). GTPase signaling: bridging the GAP between ARF and Rho. Curr. Biol. 12: R360-R362.

Schekman R and Orci L. (1996). Coat proteins and vesicle budding. Science 271: 1526-1533.

Schlunck G, Damke H, Kiosses WB, Rusk N, Symons MH, Waterman-Storer CM, Schmid SL, and Schwartz MA. (2004). Modulation of Rac localization and function by dynamin. Mol. Biol. Cell 15: 256-267.

Schmidt A and Hall MN. (1998). Signalling to the actin cytoskeleton. Annu. Rev. Cell. Dev. Biol. 14: 305-338.

Schmidt A and Hall A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. 16: 1587-1609.

Schmoranzer J and Simon SM. (2003). Role of microtubules in fusion of post-Golgi vesicles to the plasma membrane. Mol. Biol. Cell 14: 1558-1569.

Schultz J, Milpetz F, Bork P, and Pointing CP. (1998). SMART, a simple modular architecture research tool: identification of signalling domains. Proc. Natl. Acad. Sci. USA 95: 5857-5864.

Schwamborn K, Weil R, Courtois G, Whiteside ST, and Israel A. (2000). Phorbol esters and cytokines regulate the expression of the NEMO-related protein, a molecule involved in a NF-kappa B-independent pathway. J. Biol. Chem. 275: 22780-22789.

Schweitzer JK, and D'Souza-Schorey C. (2002). Localization and activation of Arf6 GTPase during cleavage furrow ingression and cytokinesis. J. Biol. Chem. 277: 27210-27216.

Seabra MC and Coudrier E. (2004). Rab GTPases and myosin motors in organelle motility. Traffic 5: 393-399.

Seabra MG, Mules EH, and Hume AN. (2002). Rab GTPases, intracellular traffic and disease. Trends. Mol. Med. 8: 23-30.

Shapiro L, Fannon AM, Kwong PD, Thompson A, Lehman MS, Grübel G, Legrand J-F, Als-Neilsen J, Coleman DR, and Hedrickson WA. (1995). Structural basis of cell-cell adhesion by cadherins. Nature 374: 327-337.

Shogomori H and Futerman AH. (2001). Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft-independent mechanism. J. Biol. Chem. 276: 9182-9188.

Shorter J and Warren G. (2002). Golgi architecture and inheritance. Annu. Rev. Cell Dev. Biol. 18: 379-420.

Simon V, Swayne T, and Pon L. (1995). Actin-dependent mitochondrial motility in mitotic yeast and cell free systems: identification of a motor activity on the mitochondrial surface. J. Cell Biol. 130: 345-354.

Small JV, Isenberg G, and Celis JE. (1978). Polarity of actin at the leading edge of cultured cells. Nature 272: 638-639.

Smith M, Simo V, O'Sullivan H, and Pon L. (1995). Organelle-cytoskeletal interactions: actin mutations inhibit meiosis-dependent mitochondrial rearrangement in the budding yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell 6: 1381-1396.

Soldati T, Shapiro AD, Svejstrup AB, and Pfeffer SR. (1994). Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. Nature 369: 76-78.

Stacey DW and Kung HF. (1984). Transformation of NIH 3T3 cells by microinjection of Ha-ras p21 protein. Nature 310: 508-511.

Stroissnigg H, Repitz M, Miloloza A, Linhartova I, Beug H, Wiche G, and Propst F. (2002). FIP-2, an IkappaB-kinase-gamma-related protein, is associated with the Golgi apparatus and translocates to the marginal band during chicken erythroblast differentiation. Exp. Cell Res. 278: 133-145.

Symons MH and Mitchison TJ. (1991). Control of actin polymerization in live and permeabilized fibroblasts. J. Cell Biol. 114: 503-513.

Tague SE, Muralidharan V, and D'Souza-Schorey C. (2004). ADP-ribosylation factor 6 regulates tumor cell invasion through the activation of the MEK/ERK signaling pathway. Proc. Natl. Acad. Sci. USA 101: 9671-9676.

Takahashi K, Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, and Takai Y. (1997). Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. J. Biol. Chem. 272: 23371-23375.

Takai Y, Sasaki T, and Matozaki T. (2001). Small GTP-binding proteins. Physiological Reviews 81: 153-208.

Takeichi M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science 251: 1451-1455

Takeichi M. (1993). Cadherins in cancer: implications for invasion and metastasis. Curr. Opin. Cell Biol. 5: 806-811.

Tall GG, Barbier MA, Stahl PD, and Horazdovsky BF. (2001). Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev. Cell 1: 73-82.

Tarricone C, Xiao B, Justin N, Walker PA, Rittinger K, Gamblin SJ and Smerdon SJ. (2001). The structural basis of Arfaptin-mediated cross-talk between Rac and Arf signaling pathways. Nature 411: 215-219.

Terbush DR, Maruice T, Roth D, and Novick P. (1996). The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. EMBO J. 15: 6483-6494.

Thompson CR and Bretscher MS. (2002). Cell polarity and locomotion, as well as endocytosis, depend on NSF. Development 129: 4185-4192.

Tuma PL and Hubbard AL. (2003). Transcytosis: crossing cellular barriers. Physiol. Rev. 83: 871-932.

Ullrich O, Horiuchi H, Bucci C, and Zerial M. (1994). Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature 368: 157-160.

Ullrich O, Reinsch S, Urbé S, Zerial M, and Parton R. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. J. Cell Biol. 135: 913-924.

Valencia A, Chardin P, Wittinghofer A, and Sander C. (1991). The ras protein family: evolutionary tree and role of conserved amino acids. Biochemistry 30: 4637-4648.

Vasanji A, Gosh PK, Graham LM, Eppell SJ, and Fox PL. (2004). Polarization of plasma membrane microviscocity during endothelial cell migration. Dev. Cell 6: 29- 41.

Velier J, Kim M, Schwarz C, KimT, Sapp E, Chase K, Aronin N, and DiFiglia M. (1998). Wild-type and mutant Huntingtins function in vesicle trafficking in the secretory and endocytic pathways. Exp. Neurol. 152: 34-40.

Vitale G, Rybin V, Christoforidis S, Thornqvist P, McCaffrey M, Stenmark H, and Zerial M. (1998). Distinct Rab-binding domains mediate the interaction of Rabaptin-5 with GTP-bound Rab4 and Rab5. EMBO J. 17: 1941-1951.

Wada M, Nakanishi H, Satoh A, Hirano H, Obaishi H, Matsuura Y, and Takai Y. (1997). Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. J. Biol. Chem. 272: 3875-3878.

Walch-Solimena C, Collins RN, and Novick P. (1997). Sec2p mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi vesicles. J. Cell Biol. 137: 1495-1509.

Wallace DM, LindsayAJ, Hendrick AG, and McCaffrey MW. (2002). Rab11-FIP4 interacts with Rab11 in a GTP-dependent manner and its overexpression condenses the Rab11 positive compartment in HeLa cells. Biocem. Biophys. Res. Commun. 299: 770-779.

Wang T and Hong W. (2002). Interorganellar regulation of lysosomes positioning gy the Golgi apparatus through Rab34 interaction with Rab-interacting lysosomal protein. Mol. Biol. Cell 13: 4317-4332.

Wang Y, Liu X, Biederer T, and Sudhof TC. (2002). A family of RIM-binding proteins regulated by alternate splicing: implications for the genesis of synaptic active zones. Proc. Natl. Acad. Sci. USA 99: 14464-14469.

Wanker EE, Rovira C, Scherzinger E, Hasenbank R, Wälter S, Tait D, Colicelli J and Lehrach H. (1997). HIP-I: a Huntingtin interacting protein isolated by the yeast two-hybrid system. Hum. Mol. Gen. 6: 487-495.

Ward SG and Westwick J. (1998). Chemokines: understanding their role in T-lymphocyte biology. Biochem. J. 333: 457-470.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, and Rothman JE. (1998). SNAREpins: minimal machinery for membrane fusion. Cell 92: 759-772.

Welch MD, Holtzman DA, and Drubin DG. (1994). The yeast actin cytoskeleton. Curr. Opin. Cell Biol. 6: 110-119.

White J, Johannes L, Mallard F, Girod A, Grill S, Reinsch S, Kellerp, Tzschaschel B, Echard A, Goud B, and Stelzer EH. (1999). Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. J. Cell Biol. 147: 743-760.

Wilson AL, Erdman RA, Castellano F, and Maltese WA. (1998). Prenylation of Rab8 GTPase by type I and type II geranyl-geranyl transferases. Biochem. J. 333: 497-504.

Wittmann T and Waterman-Storer CM. (2001). Cell motility: can Rho GTPases and microtubules point the way? J. Cell Science 114: 3795-3803.

Wodarz A. (2002). Establishing cell polarity in development. Nat. Cell Biol. 4: E39-E44.

Wu X, Wang F, Rao K, Sellers JR, and Hammer III JA. (2002). Rab27a is an essential component of melanosome receptor for myosin Va. Mol. Biol. Cell 13: 1735-1749.

Xu L, Frankel P, Jackson D, Rotunda T, Boshans RL, D'Souza-Schorey C, and Foster DA. (2003). Elevated phospholipase D activity in H-Ras- but not K-Ras- transformed cells by the synergistic action of RalA and ARF6. Mol. Cell. Biol. 23: 645-654.

Yamanaka T, Horikoshi Y, Suzuki A, Sugiyama Y, Kitamura K, Maniwa R, Nagai Y, Yamashita A, Hirose T, Ishikawa H, and Ohno S. (2001). PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. Genes Cells 6: 721-731.

Yamochi W, Tanaka K, Nonaka H, Maeda A, Musha T, and Takai Y. (1994). Growth site localization of Rho1 small GTP-binding protein and its involvement in bud formation in *Saccharomyces cerevisiae*. J. Cell Biol. 125: 1077-1093.

Yang B, Gonzalez L, Prekeris R, Steegmaier M, Advani RJ, and Scheller RH. (1999). SNARE interactions are not selective- Implications for membrane fusion specificity. J. Biol. Chem. 274: 5649-5653.

Yeaman C, Grindstaff KK, and Nelson WJ. (1999). New perspectives on mechanisms involved in generating epithelial cell polarity. Physiological Reviews 79: 73-98.

Zeitlin S, Liu J-P, Chapman D, Papaioannou V, and Efstratiadis E. (1995). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington disease gene. Nat. Med. 11: 155-162.

Zeng X, Ren M, Gravotta D, De Lemos-Chiarandini C, Lui M, Erdjument-Bromage H, Tempst P, Xu G, Shen TH, Morimoto T, Adesnick M, and Sabatini DD. (1999). Proc. Natl. Acad. Sci. USA 96: 2840-2845.

Zerial M and Huber LA. (1995). Guidebook to the small GTPases. Oxford Univ. Press, Oxford.

Zerial M and McBride H. (2001). Rab proteins as membrane organizers. Nature reviews Molecular cell biology 2: 107-119.

Zhang FL and Casey PJ. (1996). Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. 65, 241-269.

Zhang Q, Calafat J, Janssen H, and Greenberg S. (1999). ARF6 is required for growth factor- and rac-mediated membrane ruffling in macrophages at a stage distal to rac membrane targeting. Mol. Cell. Biol. 19: 8158-8168.

Zheng Y, Bender A, and Cerione RA. (1995). Interactions among proteins involved in bud-site selection and bud-site assembly in *Saccharomyces cerevisiae*. J. Biol. Chem. 270: 626-630.

Zheng Y, Zangrilli D, Cerione RA, and Eva A. (1996). The plextrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting. J. Biol. Chem. 271: 19017-19020.