THE ROLE OF THE SMALL GTPASE RAB14 IN APICAL PROTEIN
TRAFFIC AND MAINTENANCE OF CELL POLARITY

by

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The establishment and maintenance of cell polarity during development is an active process that requires specific protein sorting and targeting to apical and basolateral regions of the cell. Our lab has identified an apical early endosomal marker, endotubin, in developing rat intestine, which we have used to label specialized apical endosomal tubules, and to probe for components of the apical sorting machinery. Studies with endotubin have implicated the small GTPase Rab14 as part of the sorting machinery for apical targeting. The current work pursues further study of the interaction between Rab14 and endotubin, as well as the role for Rab14 in the establishment of cell asymmetry. Interestingly, even nonpolarized cells may utilize polarized trafficking components for proper sorting and dynamics of endotubin.
**INTRODUCTION**

**Endocytosis and Protein Trafficking**

Endocytosis is a cellular process during which eukaryotic cells internalize molecules on their surfaces, as well as macromolecules in the extracellular milieu, by forming invaginations in the plasma membrane which “pinch off” to release the membrane and its contents into the cytoplasm (Figure 1). These endocytosed molecules, within their membrane sacs (“vesicles”), undergo sorting and are directed to their next destination within the cell (Mukherjee et al., 1997). Both sorting and targeting occur within a series of morphologically and functionally heterogeneous compartments, called endosomes, which are arranged throughout the cell. One population of endosomes, the “sorting endosomes” are found at the periphery of the cell. These endosomes contain ligands and receptors that have been endocytosed and require further sorting to direct them to their ultimate intracellular destinations or to return, or recycle, them back to the plasma membrane (Dunn et al., 1989; Sheff et al., 1999). “Recycling endosomes” are a tubulovesicular population of endosomes, more perinuclear in localization and otherwise distinct from the sorting endosomes. Recycling endosomes are the major site of recycling of receptors and lipids to their membrane of origin (Dunn et al., 1989; Mayor et al., 1993).

Protein transport and membrane traffic is a highly dynamic process which requires interchange of vesicles between many different membrane domains within the cell. Yet the various endosomal compartments are able to maintain their biochemical and functional
heterogeneity even in the presence of these rapid and high-volume membrane movements. The identities of these compartments are preserved in part by the molecules that gain access to, and traffic through them, as well as the presence of a unique suite of small GTPases and their related effectors for each membrane domain (Bottger et al., 1996; Mohrmann and van der Sluijs, 1999).

**Protein Trafficking and Cell Polarity Establishment**

Cells in numerous tissues must attain and maintain a polarized state in order to accomplish their crucial functions throughout our bodies. A fundamental characteristic of polarized cells is their highly organized plasma membrane, which is arranged into functional regions conventionally denoted the apical and basolateral domains. These domains result from the selective transport and arrangement of molecules to very specific locations within the cell, as well as at its periphery. A classic example of a polarized tissue is the epithelium, or cellular lining, of the gastrointestinal tract. The apical domain of intestinal epithelial cells contains amino acid and sugar transporters whose proper localization is essential for cell health, impacts the overall health and function of the gastrointestinal tract, and the well-being of the entire organism as well. Membrane protein receptors also play a key role by recognizing specific extracellular molecules and mediating their import into the cell by endocytosis. Following uptake, these molecules undergo further processing, including processes such as transcytosis, or transport to the apical domain for exocytosis, to release the endocytosed molecules outside of the cell. In
this way, intestinal cells act as a protective, selective barrier, and also provide the body with essential molecules and ions necessary for life.

In order for a cell to establish and maintain a polarized state, it must be able to distinguish between proteins destined for different regions within the cell. Cellular machinery must deliver proteins to correct locations, remove damaged or old proteins and cell membrane, and retrieve proteins which function by cycling from one part of the cell to another. Protein synthesis occurs the endoplasmic reticulum. From there, nascent proteins are transported to another organelle, the Golgi complex, which modifies and processes the proteins, then packages them for export to the cell membrane or to other sites in the cell. A polarized cell must be able to differentiate between proteins destined for different membrane domains. This is accomplished by glycosylation patterns, amino acid sequence motifs in the protein itself, association with lipid rafts via modifications such as glycosylphosphatidylinositol moieties, and/or by interactions with other proteins. Proteins are sorted and sequestered into a budding vesicle, which then undergoes fission from the organelle of origin, and transports the cargo to its destination. Protein sorting such as described above occurs both in the biosynthetic pathway, during which newly-synthesized proteins traffic to the destination at which they function, and in the endocytic pathway, during which proteins are uptaken from the plasma membrane and transit through endosomes before either returning to the plasma membrane (recycling) or arriving at the lysosomes for degradation.
As described above, the key mechanism to the establishment of polarity is protein trafficking, the targeting and transport of proteins from their site of synthesis to their ultimate destinations. For example, mutations in the chlorine channel, CFTR, cause cystic fibrosis, which results from the failure of lung cells to properly sort CFTR to the apical cell membrane. The lack of a proper asymmetric distribution of this single protein across lung cells is enough to cause catastrophic alterations in the water and salt balance in the lungs, resulting in thick mucus production and severe, eventually fatal, damage to the lungs. Many other examples of the importance of cell asymmetry are seen in development, during which cells must migrate throughout the embryo to reach places in which they grow and differentiate to become organs and tissues. Migration is initiated by transduction of stimulatory signals, which cause reorganization of cells through redirection of protein trafficking and, indeed, the entire cytoskeleton, to induce cell motility in the direction of the stimulus. Upon reaching their destinations, these migratory cells must next respond to local signals that induce them to divide and undergo differentiation, including establishment of apical and basolateral membrane domains (e.g., in epithelia, neurons, etc.) in order for organs and tissues to develop properly.

Even after development, the maintenance of polarity by selective, directional protein trafficking and membrane dynamics remains crucial to proper function of the cell. For example, cancer can result from mutations in genes that control cell growth and/or organization. One possible mechanism in the development of cancer is the loss of cell polarity, due to mutations in genes that set up and maintain a polarized state or in genes function mechanistically in protein trafficking or regulation thereof (Stein et al., 2002).
Defects in polarity or protein sorting can lead to progressive dysregulation or dedifferentiation, and may even permit cells to invade other tissues, or enter the circulation, leading to metastatic growth.

In addition to cancer and other genetic diseases that result from defects in protein trafficking or cell polarity, a vast array of infectious organisms, from viruses to bacteria to eukaryotic parasites, have ingenious mechanisms whereby they are able to hijack the machinery that maintains cell polarity and use it to their own devices. It can be seen, therefore, that millions upon millions of people die each year from diseases that are directly or indirectly affected by defects in or disruption of cell polarity: from cancer to malaria. The burden, therefore, is on the medical research community to improve our understanding of the mechanisms of cell polarity and protein trafficking, both at the basic level, as well as in the context of disease, in order to develop effective therapies and improve human health worldwide.
Introduction

Our general interest in the dynamics of protein trafficking in nonpolarized cells stems from several fundamental questions about polarized versus nonpolarized cells:

- Is protein sorting and trafficking in polarized cells fundamentally different from nonpolarized cells with regard to machinery and organellar components?
- How does an “apical” (or basolateral) protein traffic in a nonpolarized cell?
- Do apical and basolateral sorting and trafficking machinery have specialized, functional roles in nonpolarized cells, parallel to those in polarized cells?

Answering these questions will contribute to the fundamental understanding of the mechanisms of protein trafficking in nonpolarized cells, as well as insights into metastatic growth and tumorigenesis, which could progress through loss of polarity and concomitant alterations in protein trafficking.

To address these questions, we used an apical protein marker, endotubin, to examine trafficking in nonpolarized normal rat kidney (NRK) cells. Endotubin is a type I integral membrane glycoprotein that is expressed in the developing rat intestine (Speelman et al.,
In polarized cells, endotubin is found on apical early endosomal membranes (Gokay and Wilson, 2000); in non-polarized cells, endotubin localizes to two different regions. The first is a peripheral, early endosomal compartment, which does not contain the basolateral marker, transferrin, or the TGN/late endosomal marker, mannose-6-phosphate receptor (M6PR). The second is a perinuclear compartment, which, at low resolution, exhibits colocalization of endotubin and transferrin (Wilson and Colton, 1997). Mutagenesis of the cytoplasmic tail of endotubin in the context of Tac antigen-endotubin chimeras (wild type and mutant) implicates a sorting role for two domains in the cytoplasmic C-terminus of endotubin: a hydrophobic domain (FDNILF) and a casein kinase II domain. Each is necessary and sufficient for apical early endosomal targeting (Gokay and Wilson, 2000).

Based on previous studies in nonpolarized NRK cells, peripheral endotubin does not colocalize with transferrin or M6PR, but some perinuclear endotubin-containing endosomes appear to carry either or both of these markers (Wilson and Colton, 1997). Our working model describes the peripheral endotubin compartment as containing components of what would, by convention, be termed “apical” sorting machinery. That is, mechanisms whereby apical proteins are sorted to the apical membranes in polarized cells may be conserved in nonpolarized cells, and serve a specific protein trafficking function in these cells as well. Based on colocalization data, the perinuclear endotubin compartment may therefore be a specific recycling compartment for endotubin and other proteins that interact with “apical” sorting machinery. Our purpose in this study was to characterize the perinuclear endotubin compartment in nonpolarized cells, and to
determine if its associated protein trafficking components are the same as those found in apical trafficking pathways in polarized cells.

Materials and Methods

Antibodies, Reagents, DNA constructs

Antibodies used in immunocytochemical assays: mouse anti-Golgi 58K Protein (Sigma-Aldrich), rabbit anti-Golgi β-coatomer protein (Affinity BioReagents, Golden, CO), mouse anti-clathrin heavy chain (Affinity BioReagents), rabbit anti-Rab11 (Transduction Laboratories, Lexington, KY, USA). A gift from Rob Parton, the wild-type hRab5awt gene was cloned into the pEGFP-C3 vector following PCR amplification of the Rab5 gene from cDNA. The Rab9 construct, a gift from Suzanne Pfeffer, was cloned into the pEGFP-C3 vector.

A full-length endotubin construct (Speelman et al., 1995) was subcloned into the pEGFP-C2 vector (BD Biosciences/Clontech, Mountain View, CA, USA), under the control of the cytomegalovirus immediate early promoter.

Cell Culture, Transfection

NRK cells, originally obtained from the ATCC (Rockville, MD), were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), glutamine, 100U/ml penicillin and 100µg/ml streptomycin under 5% CO₂ at 37°C. For transfection, cells were plated on 12mm coverslips in 24-well plates and incubated
overnight, until grown to approximately 50% confluency. Cells were transfected with the pEGFP-C2-endotubin construct using the Lipofectamine PLUS method (Invitrogen, Carlsbad, CA, USA). Transfection protocol was slightly modified from that supplied by the manufacturer in order to optimize transfection of NRK cells. For one well in a 6-well plate, the procedure was performed as follows: 1µg total DNA and 2.5µl PLUS reagent was diluted in 93.4µl Opti-MEM (Invitrogen) and incubated at room temperature for 15min. Lipofectamine reagent (3.8µl) was diluted in 93.4µl Opti-MEM, mixed with the PLUS/DNA mixture, and incubated for 15min, room temperature. Coverslips were washed twice with Opti-MEM and transferred to a fresh 6-well plate containing 640µl Opti-MEM (each 6-well plate can hold 2-3 coverslips for transfection). The DNA/Lipofectamine/PLUS mixture (~195µl) was added to each well, and the coverslips were incubated at 5% CO₂, 37°C. Six hours post-transfection, the media was aspirated and the coverslips were washed once and fed with 2ml antibiotic-free supplemented DMEM. Incubation continued for a further 14-18 hours, after which the coverslips were fixed, immunostained, and images were gathered and processed.

Immunochemistry

Transiently transfected NRK cells were incubated with 10µg/ml cycloheximide (unless otherwise noted) for 1hr, 37°C, 5% CO₂ to deplete proteins from the biosynthetic pathway. Cells were then fixed in freshly prepared 4% paraformaldehyde solution in phosphate-buffered saline (PBS), 20min, room temperature. Following fixation, cells were washed in PBS, quenched in 100µM NH₄Cl in PBS (15 min, room temperature), and then incubated in blocking buffer (0.5% saponin (Sigma-Aldrich, St. Louis, MO,
USA) and 10% FBS in PBS) for a minimum of 30min at room temperature. Cells were incubated with primary antibodies for 1-3hr, room temperature, washed in PBS, and incubated with fluorophore-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) for 45min, room temperature. Coverslips were washed in PBS, rinsed in water, mounted on slides using Aqua Poly/Mount (Polysciences, Warrington, PA, USA), and imaged by deconvolution microscopy.

**Antibody Uptake Assay**

NRK cells on coverslips were transiently transfected with the pEGFP-C2-endotubin vector and incubated for 20hr. Monoclonal anti-endotubin or anti-c-myc (control) antibody (tissue culture supernatants) were diluted in supplemented antibiotic-free DMEM and added to 6-well plates containing 2-3 coverslips of transfected cells per well. Plates were incubated for 30min in 5% CO$_2$ at 37°C. Half of the coverslips were treated with 21µM (100µg/ml) leupeptin (a protease inhibitor) during the antibody incubation and throughout the chase period to inhibit lysosomal degradation of proteins. After 30min, the media was aspirated, the cells were washed in antibiotic-free media, and the coverslips were placed in low-pH antibiotic-free supplemented DMEM (pH 6.8) to remove antibody bound to the plasma membrane. Coverslips were incubated for 0min, 30min, or 60min in 5% CO$_2$, 37°C, before being fixed in 4% paraformaldehyde (in PBS). Immunostaining was performed as above, except that the primary antibody incubation was eliminated. An anti-mouse IgG, Cy3-conjugated secondary antibody was used to probe for antibody uptake during the assay.
Note: Leupeptin-treated cells did not have the same dynamics of endotubin trafficking as untreated cells. Imaging parameters were identical for all cells for consistency and the purposes of comparison. As a result, the relative brightness of the perinuclear staining in leupeptin-treated cells made it impossible to image cells that were at the same levels of transfection as their uninhibited counterparts. Therefore, leupeptin-treated cells chosen for imaging were expressing much lower levels of endotubin to prevent saturation during imaging. Untreated cells could express extremely high levels of endotubin without causing saturation of the camera.

*Deconvolution Microscopy, Image Processing*

A DeltaVision Restoration Imaging System (Applied Precision, Issaquah, WA, USA) was used for image acquisition and deconvolution. Images were processed and merged using Adobe Photoshop software (Adobe Systems).

**Results**

To establish that the perinuclear endotubin compartment is not biosynthetic in nature, but is instead a component of an endocytic trafficking pathway, NRK cells were transfected with an endotubin-GFP construct. The cells were fixed, then immunostained with a panel of antibodies against several biosynthetic marker proteins: the coatamer component, β-COP (involved in ER-to-Golgi and intra-Golgi transport); the Golgi resident protein, G58K; and clathrin heavy chain (functions in traffic from the TGN and the plasma membrane). Endotubin did not exhibit colocalization with antibodies against any of these
molecules (Figure 2), indicating that the endotubin-positive perinuclear compartments are not part of the Golgi network. This is consistent with evidence previously published (Wilson and Colton, 1997), which demonstrated that endotubin colocalizes with newly-endocytosed ricin from its site of uptake at the plasma membrane, through the cell periphery, and finally, in the perinuclear region.

Having confirmed that the perinuclear endotubin compartment was not biosynthetic, the next step was to determine its position in the endocytic pathway, as well as to determine what aspects of the protein trafficking machinery might be associated with it. Both early and late endosomes can be found in the perinuclear region, but endotubin does not colocalize with Rab5 there (Figure 3). This indicates that the perinuclear endotubin compartment is not early endosomal. However, peripheral endotubin does colocalize with Rab5 (arrows in Figure 3), again confirming previous data indicating that the peripheral endotubin compartment also contains newly-endocytosed materials (Wilson and Colton, 1997).

In order to further characterize the endotubin-positive compartment, NRK cells were cotransfected with endotubin and Rab9-GFP, a marker of the late endosomal compartment. As shown in Figure 4, NRK cells were transiently transfected with Rab9-GFP and endotubin constructs, and then immunostained with an anti-endotubin antibody. Rab9 is predominantly perinuclear in localization. Endotubin is in its standard cytoplasmic distribution: throughout the cell periphery and concentrated staining at the perinuclear region. Little yellow is visible in any of the three representative cells shown
in the figure, indicating no colocalization between endotubin and Rab9. Interestingly, in the top row, and to a lesser extent in the middle row of the same figure, diffuse endotubin staining appears to fill some of the Rab9 ring-shaped structures in the perinuclear region. However, the majority of endotubin remains entirely dissociated from Rab9 in the perinuclear region, particularly the more intense, punctate endotubin structures. From this data, we can conclude that the majority of the perinuclear endotubin compartment is not late endosomal in nature.

NRK cells were also transfected with endotubin-GFP alone and stained with an anti-Rab11 antibody against endogenous Rab11. Rab11 is found in a variety of membrane compartments, including the TGN (Urbe et al., 1993), recycling endosomes, from which Rab11 appears to mediate exocytosis (Ward et al., 2005), and apical recycling endosomes in polarized MDCK cells (Casanova et al., 1999). In addition, E-cadherin transits through Rab11-positive structures as it travels to the basolateral plasma membrane (Lock and Stow, 2005). In Figure 5, Rab11 staining is clearly distinct from endotubin staining, both in the cell periphery and in the perinuclear region. Therefore, it can be concluded that the endotubin compartment does not traffic with Rab11, which is consistent with the lack of colocalization of endotubin with transferrin (Wilson and Colton, 1997). These data indicate that in nonpolarized cells, endotubin traffics distinctly from membrane proteins that also traffic separately from endotubin in polarized cells.

The next step in our characterization of the endotubin-positive perinuclear compartment was to determine whether it is a recycling compartment (albeit Rab11 negative). That is,
we wanted to determine whether endotubin was trafficked from peripheral endosomes to the perinuclear region for subsequent return to the plasma membrane. A role for the perinuclear endotubin compartment in recycling would indicate that, even in nonpolarized cells, protein processing follows different pathways depending on basolateral and apical sorting signals. Such results would have an important impact on the current thinking about protein dynamics in nonpolarized cells, challenging the notion that nonpolarized cells do not sort proteins based on motifs that in polarized cells would direct them to apical or basolateral domains. Rather, it is thought that “apical” and “basolateral” proteins do not undergo segregated sorting or trafficking in nonpolarized cells; moreover, the maintenance of separate subcellular localization and sorting pathways for these proteins is not thought to be essential to their normal biology.

To track the dynamics of endotubin in nonpolarized cells, a recycling assay was designed in which NRK cells were transfected with an endotubin-GFP fusion construct, incubated with an anti-endotubin antibody, and fixed at various timepoints. Our rationale was that, based on previous knowledge that endotubin cycles from the plasma membrane to the perinuclear region, anti-endotubin antibodies added to tissue culture media would bind the extracellular portion of endotubin at the plasma membrane. The cells would be incubated with anti-endotubin antibody to allow antibody uptake by virtue of its binding to endotubin. Fixation at various timepoints following washing the cells (to remove antibody from the media) and immunostaining with a fluorophore-conjugated secondary antibody permits visualization of only the endocytic portion of endotubin’s recycling pathway. The assay is flexible, given the ability to select different timepoints after
incubation with the antibody (for example, longer incubations could be expected to permit visualization of endotubin at later stages in the pathway, while shorter incubations would result in visualization of more membrane-proximal portions of the endotubin recycling pathway). This antibody uptake technique is useful, therefore, since it provides a simple method for selectively staining recently endocytosed endotubin and following its transit through the cell over time.

Anti-endotubin antibody (or an appropriate control antibody) treatments were conducted in the presence and absence of leupeptin, a protease inhibitor that functions to inhibit lysosomal protein degradation. The leupeptin controls were critical because recycling of endocytosed proteins (during which the antibody first appears at the plasma membrane and peripheral endosomes, gradually shifts to the perinuclear region, and eventually disappears from the perinuclear region as it recycles to the periphery) could look very much like endocytosis followed by lysosomal degradation (early membrane and peripheral localization gradually shifts to perinuclear localization, followed by disappearance as the protein reaches late endosomes and lysosomes where it undergoes proteolytic degradation). Therefore, if endotubin becomes enriched in the perinuclear region, then gradually decreases in signal intensity, even in the presence of a lysosomal inhibitor, the trafficking would be interpreted as recycling. However, results incompatible with a recycling interpretation might indicate that 1) recycling is not the function of the perinuclear endotubin compartment, or 2) other factors are complicating the issue, such as abnormal diversion of endotubin into the lysosomal degradative
pathway due to antibody crosslinking, overexpression due to high-efficiency transfection, cell-to-cell variation, and/or other issues or limitations of the assay.

The antibody uptake assay did not yield conclusive results regarding the trafficking of endotubin (Figure 6). Cells treated with leupeptin exhibited a gradual, dramatic increase in endotubin labeling in the perinuclear region. However, cells that were not treated with leupeptin showed decreased endotubin labeling over time. From this data, it appears that anti-endotubin antibody accumulates in the perinuclear region in the presence of leupeptin, but not in untreated cells. This does not support the recycling hypothesis for the perinuclear compartment, but rather that the endosomes are part of the degradative pathway. However, other factors could be confounding the issue, such that recycling is not detectable by this assay.

Another possibility is that the anti-endotubin antibody induces cross-linking, which causes artifactual trafficking of endotubin to the lysosomes. If this is indeed the case, then papain cleavage to yield Fab fragments of the anti-endotubin antibody could be attempted in order to prevent immune complexes from trafficking in a manner different from that of unbound endotubin.

**Discussion and Future Directions**

In nonpolarized cells, endotubin traffics from the plasma membrane to the perinuclear region via a peripheral early endosomal compartment. The role of the perinuclear
compartment in endotubin recycling remains unelucidated, though it is clear that the perinuclear compartment is not early endosomal (no colocalization with Rab5-GFP), late endosomal (no colocalization with Rab9-GFP), or recycling by Rab11 (no colocalization with endogenous Rab11). Endotubin antibody uptake experiments indicated that lysosomal degradation is a likely target of antibody-endotubin complexes, but does not conclusively address the question of recycling of endotubin in nonpolarized cells.

Several approaches could be taken to clarify the perinuclear endotubin compartment's identity. First, it would be important to conclusively identify the endotubin-positive perinuclear compartments in leupeptin-treated cells as lysosomes. This could be easily accomplished by immunocytochemical staining with lysosomal markers (e.g., LysoTracker or LysoSensor dyes, Molecular Probes, Eugene, OR, USA) or antibodies against lysosomal proteins (e.g., cathepsins or other proteases). In addition, dual uptake assays with fluorescently-labeled LDL (which traffics to the lysosomes) and an anti-endotubin antibody could demonstrate whether endotubin is recycling (no colocalization between LDL and endotubin at late time points) or shares the degradation pathway that LDL takes to the lysosomes.
**Figure 1. The gut epithelium is an example of a polarized monolayer.**

In a schematic model of the epithelium lining of the gut, apical (red, with microvilli) and basolateral (blue) domains are labeled. Green ovals are indicative of junctional proteins. In cell A, the red arrow indicates the general movement of vesicles carrying proteins within the apical biosynthetic pathway, and the blue arrow indicates the general basolateral biosynthetic pathway. In cell B, the arrows represent apical (red arrow) and basolateral (blue arrow) recycling pathways. In cell C, the yellow arrow represents apical-to-basolateral transcytotic traffic, while cell D shows basolateral-to-apical transcytosis.
Figure 2. Perinuclear endotubin does not colocalize with components of the biosynthetic pathway.
NRK cells, transiently transfected with endotubin-GFP (green, left column), were fixed, then stained with antibodies against several Golgi-associated proteins (middle column). In the perinuclear region, endotubin-GFP does not colocalize with (A) clathrin heavy chain, (B) beta-COP, or (C) G58K. Merged images are shown in far right column. N, nucleus. Bars equal 10µm.
Figure 3. Endotubin colocalizes with the early endosomal marker Rab5 in the periphery, but does not colocalize with Rab5 in the perinuclear region. NRK cells were transfected with Rab5-GFP and endotubin, then immunostained for endotubin. The left panel shows endotubin (green). The middle panel shows Rab5-GFP (red), and the right panel is a merged image. Arrows indicate areas of colocalization of Rab5 and endotubin in peripheral early endosomes. N, nucleus. Bar equals 20μm.
Figure 4. Endotubin does not colocalize with Rab9 in NRK cells.
Cells were cotransformed with Rab9-GFP and endotubin constructs, then immunostained for endotubin. Left column, endotubin (green). Middle column, Rab9-GFP (red). Right column, merged image. Each row shows a different NRK cell, with varying expression levels of Rab9 and endotubin. Arrows indicate green endotubin staining “within” red Rab9 structures (top two rows). N, nucleus. Bar equals 15µm.
Figure 5. Rab11 does not colocalize with endotubin in the periphery or in the perinuclear region.
NRK cells were transfected with endotubin-GFP and immunostained for endogenous Rab11. The left panel shows endotubin-GFP. The middle panel shows Rab11, and the right panel is a merged image. N, nucleus. Bar equals 10µm.
Figure 6. Antibody uptake assay.
Cells were transfected with endotubin-GFP, then incubated with anti-endotubin antibody for 30min, 37°C. Cells were chased with antibody-free media for 0min or 60min, then fixed and immunostained with secondary antibody. The top row of cells was not treated with leupeptin; the bottom row was treated with 21µM leupeptin to inhibit lysosomal protein degradation. Cells were imaged and processed identically. N, nucleus. Bar equals 10µm.
CHAPTER II: RAB14 AND APICAL MEMBRANE TRAFFIC

Introduction
Endotubin is a useful tool for studying protein trafficking in polarized and nonpolarized cells for several reasons. First, when endogenously expressed, it localized to a very specific region in the neonatal rat ileal epithelium: apical endosomes (Speelman et al., 1995; Wilson et al., 1987). It can therefore be used to tease apart various apical sorting mechanisms by comparing the localization of endotubin with other apical proteins. We have identified specific motifs within the short (36aa) cytoplasmic tail of endotubin that are necessary and sufficient to achieve proper apical trafficking (Gokay and Wilson, 2000; Gokay et al., 2001). Moreover, the endotubin cytoplasmic tail is sufficient to redirect a chimeric molecule (e.g., extracytoplasmic and transmembrane domains of Tac fused to the endotubin cytoplasmic domain (Gokay and Wilson, 2000)) to the apical domain. This implies that all the basic protein-protein interactions that are necessary for proper apical targeting of endotubin are present in its cytoplasmic domain. Therefore, the endotubin cytoplasmic domain can be used on its own to probe for other proteins involved in protein sorting and trafficking.

Large gaps still remain in the understanding of how proteins are recognized, packaged, and correctly shuttled to their destinations. Many different types of protein “machinery” have been implicated in various steps of the process. Here, we will focus on the small GTPases. Small GTPases function as molecular switches that regulate various cellular processes, including membrane trafficking events and signal transduction. Named for
their inherent GTPase activity, small GTPases cycle from an inactive, GDP-bound state, to an active, GTP-bound state. Effectors specific to each particular small GTPase regulate this cycle. For example, guanine-nucleotide exchange factors (GEFs) bind the inactive, GDP-bound GTPase, and catalyze the exchange of GDP for GTP, resulting in an activated, GTP-bound form. Another class of proteins, the GTPase-activating proteins (GAPs), increase inherent GTPase activity, which catalyzes the switch from the GTP-bound, active form of the small GTPase, to the GDP-bound, inactive form. Disruption and/or dysregulation of small GTPase cycling or effector function can lead to a variety of disease states, including cancer, birth and developmental defects, mental and neurological disorders, and metabolic disorders.

Small GTPases are grouped into the superfamily of Ras small GTPases, which has been subdivided into the Ras, Rho/Rac, Rab, Ran, and Arf families based on function and phylogeny. Small GTPases are expressed ubiquitously, and are essential for normal cell function. Over 60 Rab proteins have been identified to date, making the Rabs the largest family within the Ras superfamily. Recent research has implicated Rab protein involvement in a plethora of cellular events, such as cytoskeletal dynamics, fusion between transport vesicles and target membranes, and organellar structure. Although it is known that specific Rab proteins are localized to the cytoplasmic surface of distinct cellular compartments, their functional role in regulating membrane trafficking events is still not fully understood.
A yeast two-hybrid assay with wild-type endotubin cytoplasmic tail as bait was conducted with the intention of identifying components of the apical protein sorting and trafficking machinery, such as rabs and rab effectors. One prey plasmid isolate contained cDNA encoding the Rab14 N-terminus. The validity of the interaction was confirmed in yeast (our unpublished data), so we next began to study the function of Rab14 in mammalian cells.

Rab14 point mutants were generated that effectively “lock” the protein into either its active GTP-bound form (Rab14-Q70L), or its inactive GDP-bound form (Rab14-S25N). Transfection of Rab14-S25N-GFP fusion constructs into MDCK cells induces misprocessing of apical marker proteins. For example, the VIP/Mal proteolipid, a component of lipid rafts with a role in apical membrane trafficking, is retained in the perinuclear region in the context of Rab14-S25N-GFP overexpression in MDCK cells (Hernandez-Deviez et al., unpublished). Like VIP/Mal, gp135 (an apical PM marker) also exhibits perinuclear retention upon Rab14-S25N-GFP transfection of MDCK cells. A slightly different defect is seen for endotubin; it becomes mislocalized to the basolateral plasma membrane when the inactive form of Rab14 is present (Hernandez-Deviez et al., unpublished). Regardless of the consequences to apical targeting in the presence of Rab14-S25N, basolateral marker proteins are unaffected by the Rab14-S25N mutation. For example, the transcytotic transferrin receptor showed a distribution in Rab14-S25N-GFP-transfected MDCK cells that was not significantly different from the Rab14wt-GFP control-transfected cells (Hernandez-Deviez et al., unpublished).
Clearly, Rab14 appears to play an important and specific role in apical trafficking, but its exact function requires further clarification. This study presents the results of a yeast two-hybrid assay using a portion of Rab14 for bait, and describes possible implications for protein-protein interactions from these experiments.

**Materials and Methods**

*Reagents, Primers, DNA Constructs*

The pGBK7-Rab14-70L construct was made by cloning Rab14-70L (as previously described (Hernandez-Deviez et al., unpublished)) into the EcoRI-BamHI site of the pGBK7 vector (Matchmaker, Clontech, BD Biosciences). The three Rab14 constructs were generated by PCR. The Rab14-1 construct corresponded to amino acid residues 18-57 (5’ primer: GAGGAATTCTG GGGATATGGG AGTAGGA; 3’ primer: GGGGGATCCG CCACTAACTT CAATTAT). The middle construct, Rab14-2, corresponded to amino acid residues 63-152 (5’ primer: GGAATTCAGATTGGAATAC AGCAGGAC; 3’ primer: GCCGGATCCT TCAAGGAACA ATAAGCCG). The C-terminal construct, Rab14-3, corresponded to amino acid residues 178-447 (5’ primer: CCGGAATTCTG CAAAAACGGG AGAG; 3’ primer: GAATGGATCC TAGCAGCCAC AGCCTT).

*Yeast Two-Hybrid cDNA Library Construction*

A GAL4-based yeast two-hybrid system (Matchmaker, Clontech, BD Biosciences) was employed to isolate cDNAs whose encoded proteins demonstrated binding with a central
portion of the Rab14 protein (pGBK7-Rab14-2). For the screen, a cDNA library was generated from mRNA isolated from adult mouse kidney tissue (a gift from the Carol Gregorio lab, University of Arizona, Tucson, Arizona, USA).

Analysis of Yeast Two-Hybrid Sequences and Amino Acid Motifs

Yeast prey isolate sequences were analyzed using NCBI tools including BLAST and ORFFind and CCD to identify open reading frames, translations of DNA sequences, and conserved protein domains. ClustalW analysis was used to align Rab2, Rab4, and Rab14 amino acid sequences. Motif searches were conducted with MEME (Bailey and Elkan, 1994) and MAST (Bailey and Gribskov, 1998), which predict possible common or conserved motifs among sets of amino acid sequences.

Results

As discussed above, Rab14 has been shown to interact with the cytoplasmic domain of the apical early endosomal marker, endotubin, and appears to be necessary for its proper localization. Further validation of the endotubin-Rab14 interaction confirmed that the wild type endotubin cytoplasmic domain binds Rab14 in yeast (Figure 7). The small GTPase Rab14 therefore seems to play a role in the regulatory machinery for apical targeting in polarized cells.

Rab14-endotubin interaction experiments in yeast and mammalian cells indicate that an intact CKII site in the endotubin tail appears to play an important functional role in the
Rab14-endotubin interaction, as well as in proper localization of endotubin. Single mutants of either the hydrophobic domain or the casein kinase II (CKII) site in the endotubin tail are not individually sufficient to abrogate the interaction (Figure 7). Double mutants of the hydrophobic domain and the CKII site in the endotubin tail cause ectopic targeting of endotubin to the basolateral plasma membrane in MDCK cells. In yeast, these same endotubin double mutants do not bind to Rab14 (Hernandez-Deviez et al., unpublished; Figure 7).

To further understand and examine the role of Rab14 in apical sorting, we generated a full-length construct of Rab14-Q70L for a yeast two-hybrid assay. Initial attempts with this bait construct in the yeast two-hybrid assay met with failure. It is unclear whether the pGBKT7-Rab14-Q70L bait construct was detrimental to yeast cell growth, or whether other issues were responsible for our lack of success. Following these difficulties, we decided to develop several smaller Rab14 bait constructs for yeast two-hybrid use. After analysis of the domain structure of Rab14, three fragments of the Rab14 wild-type gene were designed, each containing regions of high conservation across Rab families and subfamilies (Figure 8), as well as regions of hypervariable amino acid sequence, in attempt to maximize the likelihood of isolation of interesting, specific Rab14-interacting proteins from the cDNA library (Figure 9). These domain constructs were made by PCR, followed by cloning of the gene fragments into the pGBKT7 vector. Of the three Rab14 fragments that were generated, the N-terminal and C-terminal constructs showed weak autoactivation, with growth on restrictive media after 5 days after transformation with empty control prey vectors. Only one of the three Rab14 constructs, which comprised the
central portion of the Rab14 amino acid sequence (90aas), passed the autoactivation test (no growth on restrictive media upon cotransformation with an empty prey vector) and was deemed suitable for use as a yeast two-hybrid bait construct. We used this central portion of the Rab14 gene in a yeast two-hybrid assay to screen an adult mouse kidney cDNA library.

Yeast Two-Hybrid Prey Sequences

A variety of genes were identified from the screen with the Rab14-middle construct. Among the cDNAs isolated in the screen, several prey vectors encode apically-targeted proteins (Table 1, Table 2). We will deal with each of the most interesting proteins individually. Refer to Tables 1 and 2 for amino acid sequences and other information about the prey isolates.

Attractin/Mahogany protein

Attractin is a major component of the melanocortin signaling pathway which drives pigmentation and also plays a major role in body weight regulation and obesity. A Type I membrane protein expressed in many organs, attractin serves as the cell-surface receptor for the secreted protein Agouti, which has a paracrine mode of action (He et al., 2003). As an apical plasma membrane protein, attractin is an attractive candidate for a marker protein to be used in addition to, or in conjunction with endotubin, which labels apical endosomes. Therefore, it could be worthwhile to establish the Rab14-attractin interaction, in order to employ it as a tool to study apical protein trafficking. The
interaction would first be confirmed in yeast by mating or two-hybrid growth on selective media, then would be further validated by biochemical studies (immunoprecipitations, GST-pulldowns, etc) using mammalian tissue culture lysates. In addition, colocalization of attractin with Rab14 would be important to establish in polarized cell types. It could be of interest to examine whether endotubin also colocalizes with attractin in the perinuclear region, if they are expected to traffic from the TGN together in Rab14-positive vesicles. If it does traffic parallel to endotubin, attractin could also be used to study nonpolarized protein trafficking, and might help surmount some of the obstacles encountered in Chapter I of this thesis, in which we were studying the role of perinuclear compartment in nonpolarized cells.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH was isolated on four independent occasions (D2, D3, I2, N2) during the yeast two-hybrid experiment. The amino acid sequence of the open reading frame of each isolate ranged from 138-228aa long, with some overlap (see Table 1). This interaction is of particular interest because an interaction of Rab2, a very close relative of Rab14, has been documented in the literature (Tisdale, 2000; Tisdale, 2003). There is some indication that atypical protein kinase may also interact with Rab2 and GAPDH (Tisdale et al., 2004; Tisdale et al., 2003). However, no interaction between Rab14 and aPKC has been identified. It would be very interesting, however, to pursue the Rab14-GAPDH connection in the future. This could be done by the standard biochemical and yeast interaction assays. Immunocytochemical methods could be employed to determine whether the two proteins colocalize. Rab14 mutants (Q70L and S25N) could be used
with GAPDH mutant constructs (to render GAPDH inactive or to alter its suspected Rab14 binding domains) to transfect tissue culture monolayers. This would help determine whether the GAPDH interaction is a functional one, or whether it is an artifact, for example, of Rab2 and Rab14 similarity, or is otherwise an uninteresting biological interaction.

**Fatty acid synthase**

A 142aa fragment of fatty acid synthase was sequenced from the F1 prey vector. In yeast, fatty acid synthase localizes to the ER, with high concentrations in a novel subdomain of that compartment: the nuclear-vacuolar interface (Kohlwein et al., 2001). Interestingly, recent research (Rappleye et al., 2003) indicate that fatty acid synthesis may be a key upstream regulator of polarity establishment early in development, by permitting the PAR genes (particularly PAR-3 and PAR-2) to associate with the paternal nucleus upon fertilization. In *C. elegans*, the proper establishment of the anterior-posterior axis is dependent upon these early polarizing events, mediated by the PAR genes (see also Chapter III of this thesis). Moreover, RNAi of fatty acid synthase completely abolishes the polarized distribution of Par3 and is lethal, but the polarity defects can be rescued by supplementation with exogenous fatty acids (Rappleye et al., 2003). Presumably, the effects of fatty acid synthase are upon membrane composition, which affects trafficking, and therefore plays a key role in the ability for a cell to establish or maintain polarity. Notwithstanding, this protein could be interesting, and might be very important in protein trafficking and cell polarity, our interests do not lie in fatty acid synthesis and metabolism. However, it might be interesting in the future to
examine whether the Rab14 interaction with fatty acid synthase is indeed a valid one (particularly since the enzyme seems to be in the “wrong place” for a meaningful Rab14 interaction: the ER). Experiments could be done to determine whether fatty acid synthase mutants disrupt protein trafficking in a specific manner in mammalian cells, such as uptake assays with ricin, transferrin, or even pulse-chase radiolabeling to study the biosynthetic pathway in the context of fatty acid synthase mutants. However, as well as being a major criticism of the C. elegans paper cited above, a serious concern in the study of enzymes such as fatty acid synthase is that important metabolic enzymes clearly can have broad, overarching effects on the cell. This can make it very difficult to discern what is a specific, direct effect, and what is secondary to the role of the enzyme in major cellular processes.

**Cobalamin receptor/cubulin**

The cobalamin receptor/cubulin is an apical protein, the vitamin B12 receptor. Cubulin is a very large, modular protein (>3600aa's). What was identified in the yeast two-hybrid assay was a 181aa fragment which contained a portion of a cubulin domain (beginning at aa3408). This portion of the molecule is extracellular, not cytoplasmic, however. Therefore, its geography is incompatible with a Rab14 interaction, since Rab14 is cytoplasmic, and this domain is situated on the other side of the membrane. However, the cubulin-Rab14 interaction has been duplicated in a second yeast two-hybrid assay which employed Rab14-ΔCXC (the C-terminal three amino acids, CGC, which comprise a prenylation motif, were truncated to prevent possible membrane association of the bait construct) as bait. An entirely different, much smaller fragment of the cubulin was
isolated (around amino acid residue 1500), which contained another portion of a cubilin domain; however, this portion, too, is extracellular. It would be ill-advised to pursue the interaction of Rab14 with cubilin any further, since there is no functional possibility for the interaction. However, there may be some use in comparing sequences of other proteins that interact with Rab14 with cubilin, in order to determine whether there may be a particular amino acid motif that binds Rab14, albeit without functional significance.

**MAP kinase kinase 2 (MAPKK2)/MEK2**

MAPKK2 is a serine/threonine kinase. Interestingly, it also was isolated a second time in the Rab14-ΔCXC yeast two-hybrid. If it does indeed have a real interaction with Rab14, which could be tested by various biochemical assays as described above, functional possibilities for the interaction include phosphorylation of Rab14. This could be tested by Western blotting for cell lysates for phosphorylated Rab14, using phospho-specific antibodies. This would determine whether Rab14 is phosphorylated, since this piece of data is currently unknown. If so, further *in vitro* phosphorylation assays could be used to determine whether Rab14 is phosphorylated by the kinase.

**Actin**

A portion (161aa) of the actin gene was identified (clone H4). To date, no evidence was available in the literature to support a direct interaction between actin and Rab family members. Without an indication of support from the literature, we are hesitant to pursue this interaction further at the present time. However, if the Rab14-actin interaction were decided to be worth pursuit, future studies would first have to confirm the interaction in
yeast. Biochemical studies (affinity chromatography or immunoprecipitation, for example), could test for an \textit{in vitro} interaction between the two proteins. The original gene fragment from the prey vector isolate could be used to generate smaller fragments of actin, in order to map the interaction domain more specifically.

\textit{Bat3/Scythe}

HLA B-associated transcript 3 (Bat3), also called Scythe, is an apoptosis-related protein with a ubiquitin-like domain. Little useful information could be gleaned from the literature about this protein; however, the ubiquitin domain could perhaps be interesting to examine as a possible site of interaction with other proteins involved in traffic, such that Scythe could serve as a bridge between Rab14 and other membrane trafficking components. If this possibility were to be pursued, the yeast interaction would first need to be confirmed. Following this, a Scythe-GST construct could be generated and GST-pulldown experiments could be employed using cell lysates, identify Scythe binding partners (analysis of proteins isolated by the pulldown would be by mass spectometry or another proteomic approach). Immunoprecipitations could then be conducted with a Scythe construct, to see whether Rab14 coprecipitates, and to determine whether Scythe and Rab14 exist in a complex with other proteins identified in the GST-pulldown, for example.

\textit{Xylulokinase B and Aldehyde reductase}

Like fatty acid synthase (see above), XylB (xylulokinase B) is another gene that was isolated in the yeast two-hybrid (clone K1) and seems to have some role in lipid
metabolism. It also has a Hsp70-class ATPase domain. Very little information can be found about this protein in the literature. Aldehyde reductase (clone N1) is another metabolic enzyme that was identified in the screen. To date, nothing indicates that either of these proteins play a role in protein trafficking or modulation of Rabs. It therefore does not seem worthwhile to pursue their study in the context of Rab14 and polarity.

Unknown sequences
Several isolates had fairly long amino acid sequences, but BLAST searches did not yield useful sequence identification or information regarding protein function. For example, several prey had similarities to Abl-351 (clone B3), Ac1147 (clones A1, B2), and a protein related to a senescence-associated protein (clone AA1). Nothing is available in the literature to indicate whether any of these would be worth pursuit as potential Rab14 effectors or interesting interacting proteins. In addition, several prey vectors sequences showed no known homology (clones E1 and K2), or the sequence only mapped to mouse chromosomal DNA, without further annotation (H1, H2, H3, K3, Q2). None of these were selected for further study. If it were determined that characterizing any of these protein fragments might be useful in understanding Rab14 trafficking, it would first be important to confirm the interaction both in yeast and biochemically. In addition, it would be useful to attempt to clone the entire gene into an expression vector. This would provide the opportunity for study of the holoprotein, which would permit more thorough study and a better understanding of its overall structure and possible functions as a Rab14 binding protein.
**Motif Searches**

Various studies indicate that the endotubin cytoplasmic tail binds the N-terminal portion of Rab14 (not shown; Hernandez-Deviez et al., unpublished); however, this does not rule out the possibility that endotubin interacts with aspects of the central domain as well. In fact, amino acid domain analysis (using MEME and MAST motif mapping tools) indicate that the proteins identified in the yeast two-hybrid may contain conserved motifs similar to those found in endotubin, which are involved in the interaction with Rab14. This may indicate that motifs within the central domain of Rab14 are important in protein-protein interactions with proteins destined to apical membrane domains. However, none of the motifs identified by MEME and MAST analysis were predicted to be statistically significant, and were therefore pursued no further. Perhaps future combinatorial manipulation of the amino acid sequences, or analysis using other types of motif-prediction software, might shed more light on the possibility that there are common amino acid motifs found in the sequences derived from the yeast two-hybrid study.

**Hydrophobic & Casein Kinase II Motif Analysis**

A hydrophobic motif and the casein kinase II domain are shared between endotubin and aquaporin-2. A variety of other apical proteins contain one, the other, or both motifs in their cytoplasmic tails. Because we have shown that endotubin's interactions with Rab14 primarily are dependent upon intact CKII and hydrophobic domains in the endotubin tail, it is possible that this amino acid sequence, if found in other apically-destined proteins, might be a conserved apical targeting motif. Further characterization of the nature of this interaction, as well as analysis of putative Rab14-interaction motifs in other apical
proteins, could help identify a protein-protein interaction domain that regulates apical targeting of cargo proteins by virtue of binding Rab14. Further studies which utilize immunoprecipitation or GST-pulldown-type experimental designs, could help determine whether these motifs are indeed binding domains for Rab14, and whether they have functional significance in protein targeting.

**Conclusions & Future Directions**

In summary, because no proteins were identified as having an obvious role in protein trafficking or an obvious function as a Rab effector, this project was discontinued. If it were to be further pursued, the first step would be to take the yeast two-hybrid prey isolates, particularly the more interesting candidates such as cubilin, attractin, and GAPDH, and confirm the protein interactions by immunoprecipitations, GST-pulldowns, and similar types of experiments. Immunocytochemical staining to detect colocalization of Rab14 and the prey protein of interest could also be useful in determining the likelihood of a functional interaction with the prey isolates. Other important future studies include mutagenesis of the Rab14 central domain, followed by retesting interactions with cargo proteins, in order to better define the nature of these interactions, and to map the Rab14 interaction domain.

Currently in progress in the lab is another yeast two-hybrid assay, which I designed in attempt to conquer the yeast two-hybrid problems. I constructed a Rab14 full-length
construct, except that it was truncated just before the last three (C-terminal) amino acids, which comprise a dual prenylation motif to allow peripheral membrane association. Yeast cells prenylate their Rab proteins in order for them to properly associate with membranes. It is known that they are able to recognize and prenylate the CXC motif of mammalian Rabs as well. Perhaps the full-length Rab yeast two-hybrid bait constructs I used were prenylated and associated with yeast membranes, limiting the ability for yeast two-hybrid bait fusion constructs (in which Rab14 is fused to the DNA-binding domain of a transcription factor) to activate transcription upon interaction with the Rab14 portion of the fusion protein. A virtually full-length Rab is more likely to fold properly and therefore pull out more meaningful prey from a yeast two-hybrid screen than a bait that only consists of a smaller region of the gene, since this smaller piece, when translated may not be able to attain proper tertiary structure and therefore prevent the identification of many interesting Rab-interaction proteins.
Figure 7. Endotubin cytoplasmic domain interacts with Rab14.

AH109 yeast cells were cotransformed with Rab14 and endotubin tail constructs. Growth on selective media indicates Rab14-endotubin interaction. 1, Rab14wt and endotubin tail wt. 2, Rab14wt and endotubin tail mutant T1186D (phosphorylation mimic). 3, Rab14wt and endotubin tail mutant T1186A (CKII knockout). 4, Rab14wt and endotubin tail F1180A (hydrophobic domain disrupted). 5, Rab14wt and endotubin tail double mutant T1186A/F1180A.

Rab14wt binds endotubin wt in yeast AH109 strains. Single mutants of the hydrophobic domain or the casein kinase II (CKII) site in endotubin tail are not individually sufficient to abrogate the interaction (2-3, yeast growth). The double-mutant form of endotubin (F1180A/T1186A) does not bind rab14 (5, no yeast growth).
Figure 8. ClustalW 1.82 alignment of the full-length *Rattus norvegicus* Rab4a, Rab14, and Rab2 amino acid sequences.

Above the alignments are annotations identifying Rab family domains (RabF), Rab subfamily domains (RabSF), GTP-binding domains (GTPb, highlighted yellow), α-helices (α), and β-sheet (β) structures. Below the alignments, asterisks (*) indicate exact sequence matches, colons (:) indicate conserved residues, and periods (.) indicate semi-conserved residues.
Figure 9. Diagram of Rab14 fragments generated for yeast two-hybrid analysis. The P-loop, switch I, and switch II regions exhibit alterations in conformation depending on the guanine nucleotide binding state of Rab14. GTP-binding regions and Rab family and subfamily domains are shown (see key). The three black bars above the schematic of Rab14 indicate the three different yeast two-hybrid constructs that were made. The middle construct, which contains the switch II region, was the one used in the yeast two-hybrid assay.
Table 1. Yeast two-hybrid assay: isolated prey amino acid sequences and identities in alphabetical order by clone.
The bait construct was the pGBKT7-Rab14-2 construct, which consisted of a 90aa central portion of the wild-type Rab14 gene.

<table>
<thead>
<tr>
<th>Clone</th>
<th>AA sequence</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
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<td>A1</td>
<td>GCTRATLTGSACAYPTPAGAGNPLNPIRDGDRGLQLFPMNEEFPVSAGHKLALIKS LPFVHT</td>
<td>Ac1147</td>
</tr>
<tr>
<td>A2</td>
<td>GSASATHGGAPLPGTRPGASVHVRAN NSYVMVGTNFNLPSDGSAVDVHINMEQA PIQSEPRVRLMAQHMIRD TLLSRLME CRGCTQAOASPPPQTPQTVASETVAL NSQTSEPVESEAPPREPMESEEMEERP PTQTPELAPSAGPAGPAPAG</td>
<td>Bat3, Scythe; ubiquitin-like protein, regulator of apoptosis</td>
</tr>
<tr>
<td>AA1</td>
<td>WPGIRTEQQDQASCFPSAPREVSVLPEL ALGHLYRRTGPQNSPPTVPAGAG RAR</td>
<td>hypothetical protein, proprotein convertase aPC6C isoform, senescence-associated protein</td>
</tr>
<tr>
<td>B2</td>
<td>SASPNFLEGQVAFSHPRLSNNRSVMPL DVRGCARATLTGSACAYPTPAGAGNPL NPIRDGDRGLQLFPM NEEFPVSAGHKLALIKSLPFVHT</td>
<td>Ac1147</td>
</tr>
<tr>
<td>B3</td>
<td>MDYRNEYNLLLKEIEIQQMASTECPTS QATLRFYLTVPVREIIQQVTAKASEYAE KENSYPL</td>
<td>LRRGT00057, similar to Abl-351</td>
</tr>
<tr>
<td>D1</td>
<td>PGDYDVNITYGGVGSPFRVPSSDV DPSKVIAGPGLLSSCVRACIPQSFRTVDSS KAGLAPLEVRVLRGPR GLVEPVNVVDNG DGTHTVYTPSEQEGPYIVSVKENAEIFIED PR SPFKVKLPMTPYDATSKVATSGPGLSAYGV PASLLWSSPMPKAMLKCPDPDHPG GAKSYNVHDNGDGYALPSLIRHTGRY MIGVTLWGETTFLPSLPYPGPGQXXXNATXCLATGXXGNCPTXTKTEGGGGLFYWXL KTAEXEGE</td>
<td>filamin</td>
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<tr>
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<td>----------------------------------------------------------------------------</td>
<td>-------------------------------</td>
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<td>PITIFQERDPTNIKWGEAGAKEYVVVESTGVFTTVEKAGAHLLGGAKRVIISAPSADAPMFVMGVNHEKYDNSLKVSNASCTTNCLAPLAKVIHDNFGIVEGLMTTVHAITATQKTVDGPSGLWRDGRGAAQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPT</td>
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</tr>
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<td>no significant homology</td>
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<td>E2</td>
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<td>cobalamin receptor, cubulin, intrinsic factor receptor</td>
</tr>
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<td>F1</td>
<td>PGPRGTPLISPHIKWDHSQTWDVPVAEDFPNGSSSSATVYISADSPESPDHYLVDHCIDGRVIFPTGYCLVWKLARSGLSLEETPVVFENVSFHATILPTGTVALEVRLLEASHAFESDGTGLNGIVSGKYLVWED</td>
<td>fatty acid synthase</td>
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<td>H1</td>
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<td>Mus musculus chromosome 5, clone RP24-84G9</td>
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<td>H2</td>
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<td></td>
<td></td>
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<tr>
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<td>EREIVRDIEKEKLNCYVALDFEQEMATASSSSEKSYELPDQVITIGNERFRCPAEALFQPSFLGEMSCGIHETTFNSIMKCDVDIRKDLYANTVLSGGTMYGPIADRMQKEITALPSTMKIKIAPPERRYSVWIGGSILASLSTFQQMWISKQYEDES</td>
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</tr>
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<td>-------------</td>
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</tr>
<tr>
<td>I2</td>
<td>GEAGAEYVVVESTGVFTTMEMKAGAHLKG GAKRVIISAPSADAPMFVMGVNHEKYDN SLKIVSNASCCTNCLAPLAKVHDNFGIVE GLMTTVHAITATQKTVDGPSGKLWRDG RGAANNTPASTGAAKAVGKVIPELNGKL TGMAFRVPTPNVSVDLTCAKPAKLYD DIKKVVQASXGPLKGIILGYTXDQVVS CDFATPTLPPSMGLALLSMTTLSFPM GMT</td>
<td>GAPDH</td>
</tr>
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<td>EQPGCGWCTDPSNTGKGCIEGSYKGP VKMPSQAASNQVYQPOPLNSMCELDSS YRNWSFIHCAPCCQCGHSCKINQSIKE CEDLTGKHCETCISGYGDPTNGKC QPCKNCNHSCLNTNGKCFCTTGVK GDECQLCEVENRYQGGNPQLGTCYTTLL TISSPLVQEDDREYNTAINFVATPDEQN</td>
<td>attractin, mahogany protein</td>
</tr>
<tr>
<td>K1</td>
<td>GHIFCNVPDPQHYMALLCFKNGSLMREK IRDESASCSWNKFSKALGSTAMGNNGN LGFYFDVIMEITPEIGRHFRFNAEMEVS FPDGEVIRALIEGFMATRIHGAEGLGYRVM PKTKILATGGASHNKLQILADVFGAP VYVIDTT</td>
<td>XylB (xylulokinase B); Hsp70-Class ATPase domain</td>
</tr>
<tr>
<td>K2</td>
<td>WGPFLSSSRSFIL</td>
<td>no significant homology</td>
</tr>
<tr>
<td>K3</td>
<td>MAGAANLVPQAQYEYARFSCLPLAH AAMPLVSLKLAERADMLFFSGGNNLSSFY TRSTDNSTQERED</td>
<td>Mus musculus strain 129/Sv clone ct7-297f7 map X</td>
</tr>
<tr>
<td>N1</td>
<td>RGDNPFPKNADGTVYDSTHYKETWKA LEVVLAKGLVAKLSNFNSRQIDDVLSV ASVRPAVLQVECHPYLAQNELIAHCHAR GLEVTAYSLGSDDRAWHRPDEPVLEEL PVVLALAEKHGRSPAQILLRWQVQRKVI CIPKSNPSRILQNIQVFDFTSPEEMKQL DALNK</td>
<td>aldehyde reductase</td>
</tr>
<tr>
<td>Clone</td>
<td>AA sequence</td>
<td>Identity</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
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| N2    | DPTNIKWGEGAAEYVVESTGVFTTMEKA GAHLKGGAKRVIISAPSADAPMFVMGVN HEKYDNSLKIIVSNASCTTNCLAPLAKVIY
       | DNFGLVEGLMTTVHAITATQKTVDGPSG KLWRDGRGAQQIIPASTGAAKAVGKVI PELNGKLTGMAFRVPTPVNVSVDLCRLEK | GAPDH                           |
| O1    | RAERYGRAMSAASASRGWASIRDPSSS SCR                                             | no significant homology         |
| Q2    | TKVILVIYNTRQLRPKGLDTPCLAINLN                                               | *Mus musculus* clone LA9 mitochondrion |
| T1    | RDQIMHRDVKPSNILVNSRGEIKLCDFGV SGLIDSMANFVGTRSYMSPERLQGT HYSVQSDIWSMGLSLVELAIGRYIPPPPDAKELEASFGRPVVDGADGEPSVSPRP RPGRPI SGHMDSRPAMAIFELLDYIVNEP | MAPKK2 / MEK2 (S/T kinase)       |
Table 2. Yeast two-hybrid results: isolated prey amino acid identities grouped according to function.
The bait construct was the pGBK7-Rab14-2 construct, which consisted of a 90aa central portion of the wild-type Rab14 gene.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Identity</th>
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<tr>
<td>D1</td>
<td>filamin</td>
</tr>
<tr>
<td>H4</td>
<td>actin</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytoskeletal</strong></td>
<td></td>
</tr>
<tr>
<td>AA1</td>
<td>hypothetical, proprotein convertase aPC6C isoform, senescence-associated protein</td>
</tr>
<tr>
<td>E2</td>
<td>cobalamin receptor, cubulin, intrinsic factor receptor</td>
</tr>
<tr>
<td>J1</td>
<td>attractin, mahogany protein</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Apical membrane</strong></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>Bat3, Scythe; ubiquitin-like protein, regulator of apoptosis</td>
</tr>
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<td></td>
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<tr>
<td><strong>Apoptosis</strong></td>
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<td>aldehyde reductase</td>
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<td></td>
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<td><strong>Kinase</strong></td>
<td></td>
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<tr>
<td>K1</td>
<td>XylB (xylulokinase B); Hsp70-Class ATPase domain</td>
</tr>
<tr>
<td>T1</td>
<td>MAPK2 / MEK2 (S/T kinase)</td>
</tr>
<tr>
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<tr>
<td>O1</td>
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<td>Q2</td>
<td>Mus musculus clone LA9 mitochondrion</td>
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CHAPTER III: RAB14 AND THE ESTABLISHMENT
OF CELL POLARITY (ASYMMETRY)

Introduction

As has already been discussed, cell polarity, or asymmetry, is the differential distribution of proteins and subcellular structures across the cytoplasm and/or plasma membrane of a cell. The specialized plasma membrane domains or intracellular regions that are generated during polarization permit a cell to interact with many different simultaneous environmental stimuli at maximum efficiency. Both establishment and maintenance of asymmetry are critical for proper individual cell function, as well as the survival and well-being of the organism as a whole.

Traditionally, polarity establishment has been thought to occur during or after the development of a confluent monolayer, following formation of junctional complexes that induce functional isolation of membrane domains from each other. Early models were based on the concept that apical and basolateral protein sorting and trafficking machinery functions in a constitutive manner in nonpolarized cells (or cells undergoing polarization). In these models, apically-directed or basolaterally-directed proteins are properly directed to their respective domains by specific protein trafficking machinery (Rabs, SNAREs, etc), but diffuse from apical to basolateral domains, and vice versa, until the establishment of junctions. Upon reaching confluency and establishing tight junctions, apical and basolateral proteins become physically restricted to the proper membrane domains, and can no longer diffuse from their sites of delivery. As polarization progresses, the cell must reorganize other aspects of its architecture, such as
the cytoskeleton and organellar structure. Following establishment of polarity, the cell must maintain its polarized state for the duration of its life. Even during mitosis, a polarized cell must properly orient the spindle and execute cytokinesis in a manner that preserves the integrity of the epithelium, and to avoid compromising the tissue.

Recently, the discovery of a family of genes in *C. elegans* has challenged aspects of the traditional model for cell polarity. This family, the PAR serine/threonine kinases, consists of six members in *C. elegans*. Some of these act upon each other hierarchically (as a kinase cascade), while others serve to inhibit each other to prevent one membrane domain from encroaching upon the other. PAR homologs have been identified across the eukaryotic spectrum, from *Drosophila* to mammals. Fascinatingly, PAR proteins have been shown to play critical roles in many aspects of asymmetry and polarity, at both cellular (apical-basolateral identity) and organismal (anterior-posterior axis) levels. PAR proteins have been shown to affect actin cytoskeletal dynamics through the Cdc42/Rac GTPase with resulting alterations in membrane traffic and ultrastructure (Kim, 2000; Nishimura et al., 2005). It should be noted that, although the role for PAR genes in polarity has been well-established, certain aspects of the literature in this area challenge traditional models for the establishment of polarity and asymmetry. For example, the mammalian PAR-4 homolog, LKB, can induce polarity in a cell-autonomous manner (Baas et al., 2004). Even more surprisingly, LKB can induce apical membrane in a single-cell suspension – without the presence of a substrate for the cell to rest on (Baas et al., 2004; Spicer and Ashworth, 2004).
PAR Proteins and Apical Membrane Identity

A heterotrimeric complex, composed of Par3, Par6, and the atypical lambda isoform of protein kinase C (aPKC\(\lambda\)), appears to play a critical role in the establishment of polarity in epithelia by defining the apical membrane domain. The Par3/Par6/aPKC complex has the earliest role of the PAR hierarchy in mammalian cell polarity establishment, and in fact may be one of the earliest players overall in the development of cellular asymmetry (Gao et al., 2002; Horne-Badovinac et al., 2001; Hurd et al., 2003; Nagai-Tamai et al., 2002; Ohno, 2001; Straight et al., 2004; Suzuki et al., 2002). Cell-cell contact appears to be the signal which induces the recruitment of the complex to the apical plasma membrane, which then activates a cascade of other signals, resulting in recruitment of other junctional proteins and reorientation of membrane traffic.

Various members of the Par3/Par6/aPKC complex have been studied individually, giving indications of the diverse types of interactions and signaling in which they are involved. For example, Par3 interacts with JAM, a cell-cell adhesion molecule (Ebnet et al., 2003; Itoh et al., 2001). Moreover, knockdown of Par3 results in disruption of tight junction assembly, and defects in cell polarity (Chen and Macara, 2005). Dominant-negative forms of aPKC also cause disruption of tight junctions (Suzuki et al., 2001). This lends support to the concept that the Par3/Par6/aPKC complex functions upstream of junctional protein recruitment to the cell-cell interface. Adding to the complexity, Par6 interacts with yet another trimeric protein complex, Crumbs/PALS/PATJ, which is also extremely important in the definition of the apical membrane (Hurd et al., 2003) and maintenance of polarity. Par6 also interacts with the active form of Cdc42, which has implications for
regulation of the actin cytoskeleton (and therefore membrane dynamics as well as cell ultrastructure) during the process of polarization (Peterson et al., 2004). In Drosophila, aPKCλ mutants exhibited gross developmental defects in their gut tubes, while knockouts experienced severe defects in cell polarity and spindle orientation (Horne-Badovinac et al., 2001). aPKC has also been shown to interact with the N-terminus of Rab2 (Tisdale, 2003). This indicates that the complex might have direct interactions with protein trafficking machinery in addition to more indirect effects, such as regulation of the actin cytoskeleton.

The Par3/Par6/aPKC complex is not limited to acting as a holocomplex, in which different regions of the assemblage interact with various effectors. Indeed, Par3, Par6, and aPKC not only bind to each other, but they appear to act on and modify each other in quite complicated ways. For example, aPKC phosphorylates Ser827 of Par3 (Nagai-Tamai et al., 2002). In addition, both Cdc42/Rac1 and Par6 increase the kinase activity of aPKC, which regulates its kinase activity on Par3 (Chen and Macara, 2005). This demonstrates that factors both extrinsic to and intrinsic to the complex affect its dynamics and function in a tightly regulated fashion. Sensitive, rapid self-regulation and feedback must therefore be of vital importance for the proper function of this heterotrimeric complex in the establishment of and maintainence of cell asymmetry.

Rab14 and Polarity

Research in our lab indicates a role for Rab14 in the machinery that conducts polarized protein trafficking. Rab14 appears to function at the level of the TGN; Rab14-positive
vesicles fuse with apical early endosomes as well as the plasma membrane. The dominant negative form, Rab14-S25N, accumulates in the TGN, and causes basolateral missorting of endotubin (Hernandez-Deviez et al., unpublished). The Rab14 small GTPase shows highest similarity to Rab4a (found on early endosomes) and Rab2 (pre-Golgi function) (Figure 8). As mentioned above, Rab2 interacts directly with aPKCλ by biochemical assays (Tisdale, 2003). However, Rab2 functions between the ER and Golgi apparatus, which does not seem to be a likely prospect for regulated polarized protein trafficking. Therefore, it is possible that the biochemical evidence supporting Rab2 and aPKC interaction, albeit true, does not have a functional biological significance. Instead, perhaps Rab14 is the Rab that has the relevant interaction with aPKC, which would be a logical function for a Rab that plays a role in apical targeting. We decided to determine if Rab14 interacts with aPKC, and therefore the Par3/Par6/aPKC complex. In addition, we examined whether mutants in Rab14 affected localization of the Par complex to the apical domain as well as polarity establishment during recovery from calcium depletion. Our hypothesis was that Rab14 interacts with aPKC, but acts upstream of the Par complex, such that Rab14 dominant negative mutants impair polarity establishment.
Materials and Methods

Antibodies, DNA (Rab14 for transfections)

Antibodies used in immunocytochemical assays: rabbit anti-ZO-1 (Zymed Laboratories, Invitrogen), mouse anti-aPKC\(\lambda\) (BD Transduction Laboratories, Mountain View, CA, USA), rabbit anti-Par3 (Upstate, Charlottesville, VA, USA). The pEGFP-Rab14 and pEGFP-Rab14-S25N constructs were made as previously described (Hernandez-Deviez et al., unpublished), by cloning PCR products into the EcoRI-BamHI site of the pEGFP vector.

Cell Culture, Transfection

MDCK cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), glutamine, 100U/ml penicillin and 100\(\mu\)g/ml streptomycin under 5% CO\(_2\) at 37\(^\circ\)C. Stably transfected cells were maintained in this media plus the appropriate selective agents, G418/Geneticin (Invitrogen) and Hygromycin (Sigma-Aldrich, St. Louis, MO, USA). Stably transfected MDCK cells were plated at confluency in transwell filters in 12-well plates and incubated for 5 days (with daily feeding) to establish polarity. 4mM butyrate was added to induce expression, and plates were incubated for 7hr, 37\(^\circ\)C, 5% CO\(_2\).

Calcium switch

Calcium-free media was prepared by dialyzing FBS in PBS, then supplementing SMEM (Invitrogen) with 2% calcium-free FBS. Untransfected MDCK cells were plated at confluency on transwell filters in 12-well plates. Cells were allowed to establish polarity
for 5 days (daily feeding), then were incubated with low-calcium media for 24 hr. Cells were rinsed once with low-calcium media, then normal MDCK media (RECIPE) was added and the plates were incubated at 37°C for various time points (30 min, 60 min, 105 min of recovery). At each time point, the filter was removed to another plate, and immediately fixed in fresh 4% paraformaldehyde in phosphate-buffered saline solution, 20 min. Immunocytochemical staining was conducted as described below.

**Immunocytochemistry**

Cells were washed briefly, then fixed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS), 20 min, room temperature. Following fixation, cells were washed in PBS, quenched in 100 μM NH₄Cl in PBS (15 min, room temperature), and then incubated in blocking buffer (0.5% saponin (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS in PBS) for a minimum of 30 min at room temperature. Cells were incubated with primary antibodies for 1-3 hr, room temperature, washed in PBS, and incubated with fluorophore-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) for 45 min, room temperature. Coverslips were washed in PBS, rinsed in water, mounted on slides using Aqua Poly/Mount (Polysciences, Warrington, PA, USA), and imaged by deconvolution microscopy.
Results

As members of the same Rab subfamily, Rab2 and Rab14 share a great deal of sequence homology (Figure 10). In fact, the N-terminal 19aa of Rab2, to which the aPKC interaction has been mapped (Tisdale, 2003), is identical to Rab14 but for three residues (Figure 11), which correspond to amino acids 2, 4, and 15 of Rab2. We therefore decided to determine whether aPKCλ interacts with Rab14 as well as Rab2, since it seemed in fact more likely that Rab14 would have the functional interaction with aPKCλ, based on both proteins' subcellular localizations (aPKCλ to the junctional region at the plasma membrane, and Rab14 to the TGN and apical plasma membrane). In addition, our work implicating Rab14 in polarity establishment and apical protein trafficking lends credence to our model, in which Rab14 might interact with the Par3/Par6/aPKC complex.

To determine whether Rab14 binds aPKCλ, we did immunocytochemical staining in stably-transfected MDCK cells to look for colocalization between the two proteins. We also stained MDCK cells for Par3, to better visualize the Par3/Par6/aPKC complex. Figure 12 shows MDCK cells expressing either GFP fusion constructs of either Rab14wt, or Rab14-S25N. No colocalization with aPKCλ was observed among any of the Rab14 isoforms (including Rab14-Q70L, data not shown). Consistent with this, aPKC and Par-3 have similar staining patterns (compare middle column of Figure 13A with Figure 13B) and colocalize with each other (Figure 14, middle and bottom panels). In addition, Par-3 does not colocalize with any Rab14 isoform (not shown). Lack of colocalization does not absolutely rule out the possibility of an aPKCλ-Rab14 interaction, however: if the nature of the interaction is very rapid (i.e., a phosphorylation event), no colocalization may be
seen, although a biological interaction does occur.

Immunoprecipitation experiments were attempted with stably transfected Rab14wt-GFP MDCK cell lines. Several antibodies against GFP were used, but the immunoprecipitation was unsuccessful. However, Rab14 is notoriously difficult to work with from cell lysates and in immunoprecipitation experiments. It is possible that an interaction exists, but technical difficulties precluded discovery of any interaction by these methods.

The next attempt to determine whether Rab14-S25N disrupts localization of the Par3/Par6/aPKC complex at the junctional region was through studying stable transfectants expressing GFP constructs of Rab14wt and Rab14-S25N. Cells were grown on transwells to establish a polarized phenotype. The cells were fixed, then immunostained for aPKC and Par3. Figure 13 shows no difference between Rab14wt and the dominant negative S25N cells in the ability for members of the Par complex (Par3 and aPKC) to localize to the plasma membrane. In addition, there was no visible difference in the localization of Par3 and aPKC in Rab14-expressing cells compared to untransfected neighboring cells. Therefore, it seems that if Rab14 does interact with aPKC, it is a very subtle interaction.

Our hypothesis predicted that Rab14-S25N affects aPKC localization during the process of polarity establishment. That is, perhaps Rab14, if acting upstream to aPKC and the Par3/Par6/aPKC complex as a whole, cannot affect it once the complex has localized to
the apical junctional region. Therefore, an assay in which the cells polarize in the presence of Rab14wt or Rab14-S25N might have the necessary power to tease out a subtle or very early interaction between the Par complex and Rab14. To compare the aPKC and Par3 localization in the context of different isoforms of Rab14, transfected MDCK cells were subjected to a calcium switch assay. This experiment consists of an extended incubation in low-calcium media until the cells lose intercellular contacts and their polarized phenotype. The cells are then returned to normal culture media; at various timepoints, samples are fixed and immunostained to observe reestablishment of polarity and the localization of the Par complex. Unfortunately, the calcium switch assay seemed to have a marked negative effect on the ability for transfected MDCK cells to maintain Rab14 expression. Attempts with both stable and transient transfectants resulted in the same loss of expression during the low-calcium period, preventing any study of the effects of the Rab14 isoforms on the Par3, Par6, and aPKC with this assay.

In the experiments described above, ZO-1, a tight junctional protein, was used as another marker for polarity reestablishment. During the course of the calcium switch assays, it was observed that ZO-1 appears to reach the junctional region of the cell more rapidly than aPKCλ and Par3. ZO-1 localizes to the tight junctions as early as 15min into recovery from calcium switch (not shown), while aPKC and Par3 show a slight lag in return to the junctional region (Figure 14). In Figure 15, ZO-1 can be seen to be present at approximately 50% of cell perimeters, whereas aPKCλ is much more sparsely localized to the junctional region of polarizing cells. By 60min, ZO-1 is fully localized to the tight junctions, while aPKCλ is significantly less strongly associated with the
junctional region (Figures 14 and 15). However, by 105min, both aPKC\(\lambda\) and ZO-1 have recovered full staining at the cell junctions compared to unswitched cells (Figure 15 and data not shown). aPKC and Par3 appear to recover their polarized distributions at the same rate (Figure 14), showing very sparse junctional localization at 30min, which gradually increases by 60min, reaching full localization by 105min to the cell junctional region. This seems to be at odds with the general concept that the Par3/Par6/aPKC complex is upstream of tight junctional formation. It must be noted that the aPKC\(\lambda\) antibody and the Par3 antibody stain much more weakly than the ZO-1 antibody. This might limit the ability to visualize very small quantities of aPKC or Par3 given the lower antibody affinities for these proteins; however, the overall observations and conclusions regarding the relative localizations of Par3, aPKC, and ZO-1 during the calcium switch assay are not seriously affected by this issue.
Discussion & Future Directions

In contradiction with our hypothesis, Rab14 does not appear to interact with aPKCλ by various immunocytochemical methods. However, immunoprecipitation experimental data remained elusive. It could prove worthwhile to repeat the immunoprecipitation experiments, if better techniques are developed for biochemical manipulation of Rab14.

Another approach to the question of the aPKC-Rab14 interaction might be to clone the aPKCλ gene into a yeast vector, and conduct experiments in yeast with various Rab14 constructs. This could help establish whether the interaction is worth further study.

Given the calcium switch data in which ZO-1 “beat” aPKC and Par3 to the junctions, further study of the repolarization of MDCK cells could prove interesting, particularly since this appears to conflict with the current literature on how the Par complex induces polarity. Again, the results from this study could possibly be confounded by extreme differences in antibody affinity, which masks the true time-course of recovery of aPKC and Par3 following calcium switch.
Figure 10. ClustalW 1.82 alignment of the full-length *Rattus norvegicus* Rab4a, Rab14, and Rab2 amino acid sequences.

Above the alignments are annotations identifying Rab family domains (RabF), Rab subfamily domains (RabSF), GTP-binding domains (GTPb, highlighted yellow), α-helices (α), and β-sheet (β) structures. Below the alignments, asterisks (*) indicate exact sequence matches, colons (:) indicate conserved residues, and periods (.) indicate semi-conserved residues.
**Figure 11. Predicted aPKCλ binding site on Rab2 (brackets, colored sequence) (Tisdale, 2003).**
The N-terminus of the Rab14 is aligned under Rab2. Identical amino acid residues are bolded; the three dissimilar residues between the sequences are in plain text.

<table>
<thead>
<tr>
<th>rab2</th>
<th>MAYAYLFKYIIIIGDTGVGKSCLLQFTDKR</th>
</tr>
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<tbody>
<tr>
<td>rab14</td>
<td>MATTPYNSYIFKYIIIIGDMGVGKSCLLHQFTEKK</td>
</tr>
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</table>

aPKCλ binding
Figure 12. aPKC does not colocalize with Rab14wt or Rab14-S25N.
MDCK cells expressing Rab14wt or Rab14-S25N were grown to confluency on coverslips, then fixed and stained for aPKC. Top row, untransfected cells. Middle row, MDCK cells expressing Rab14wt. Bottom row, MDCK cells expressing Rab14-S25N.
Figure 13. Rab14S25N does not affect localization of aPKCα or Par-3.
Stable MDCK cell lines expressing Rab14wt-GFP or Rab14-S25N-GFP were plated at confluency on transwell filters and allowed to polarize. Cultures were fixed and immunostained for A, aPKCα or B, Par-3. Note no colocalization of Rab14 constructs with aPKC or Par-3. Staining at the junctional regions of cells was unaltered in the Rab14-S25N cells (arrowheads). Surrounding untransfected cells (no green) are not different from transfected cells in their levels or localization of aPKC or Par-3.
Figure 14. Calcium switch: aPKC and Par3.
MDCK cells were grown to confluency on transwell filters, then cultured for 24 hours in low Ca++ media. Cultures were switched to standard media for various timepoints, then fixed and immunostained for aPKC\(\lambda\) and Par-3. Top row: 30min post-switch. Middle row: 60min post-switch. Bottom row: 105min post-switch. Note the gradual increase in junctional accumulations of both aPKC and Par-3 until cells regain polarity by 105min.
Figure 15. Calcium switch: aPKC and ZO-1.
MDCK cells were plated at confluency on transwell filters, allowed to polarize, then cultured for 24 hours in low Ca++ media. Cultures were switched to standard media for various timepoints, then fixed and immunostained for aPKC and ZO-1. Top row: 30min post-switch. Middle row: 60min post-switch. Bottom row: 105min post-switch. Note the gradual increase in junctional accumulations of both aPKC and ZO-1 until cells regain polarity by 105min. Also note lag in the return of aPKC to the junctional level in comparison to ZO-1.
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