RAB13 REGULATES MEMBRANE TRAFFICKING BETWEEN THE TRANS-GOLGI NETWORK AND RECYCLING ENDOSONMES IN POLARIZED EPITHELIAL CELLS

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ABSTRACT

Rab13 regulates membrane trafficking between the trans-Golgi network and recycling endosomes in polarized epithelial cells

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To maintain polarity, epithelial cells continuously sort transmembrane proteins to the apical or basolateral membrane domains during biosynthetic delivery or after internalization. This thesis was designed to elucidate the molecular mechanism underlying protein trafficking. Our studies identified Rab13 as a critical GTPase regulator of biosynthetic cargos that are transported from the trans-Golgi network (TGN) to recycling endosomes before being delivered to the plasma membrane. We showed that Rab13 partially co-localizes with TGN38 at the TGN and transferrin receptors at recycling endosomes. Overexpression of dominant active or dominant negative alleles of Rab13 disrupted localization of the TGN marker, TGN38, in coverslip grown MDCK cells. Importantly, this phenotype was unique to Rab13, as compared to Rab8 and Rab10. In polarized MDCK cells, we microinjected cDNAs encoding Rab13 mutants and transmembrane cargo proteins. We found that mutations of Rab13 resulted in impaired surface delivery of cargos that normally sort to the plasma membrane through recycling endosomes (VSVG, A-VSVG, and LDLR-CT27). Rab13 mutants, however, had little effect upon cargos traveling a direct path to the plasma membrane (LDLR(Y18A), FcR, and HA). These results strongly support the hypothesis that Rab13 is involved in the transport of cargo from the TGN to the recycling endosomes.
A second part of this thesis examined the evolutionary conservation of sorting signals across different cell types. We characterized the sorting of a transmembrane protein, NgCAM that is expressed endogenously by both epithelial cells and neurons. We found that the same NgCAM cytoplasmic tail sequence (45-59) promotes enrichment in the axonal domain of neurons as well as apical localization in epithelial cells.
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A-VSVG  apical variant of vesicular stomatitis virus glycoprotein
AP     Adaptor protein
Arf    ADP-ribosylation factor
ARH    autosomal recessive hypercholesterolaemia
Arl1   ARF-like GTPase
3-AT   3-amino-1,2,4-triazole
CCVs   Clathrin-coated vesicles
CDR    complementarity-determining region
CHX    cycloheximide
COPI   coatomer protein complex-I
COPII  coatomer protein complex-II
CtBP3/BARS carboxy-terminal binding protein 3/brefeldin A-ribosylated substrate
DDO    double dropout plates
ER     endoplasmic reticulum
FcR    FcII-B2 receptor
GAP    GTPase-activating protein
GDI    GDP dissociation inhibitor
GDP    guanosine diphosphate
GEF    guanine nucleotide exchange factor
GGA    Golgi-localized, γ-ear-containing, Arf-binding protein
GPI    glycosylphosphatidylinositol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GRIP</td>
<td>Golgin-97, RanBP2α, Imh1p and trans golgi p230 conserved domain</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5′-triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HBE</td>
<td>human bronchial epithelial cell line 16HBE14o-</td>
</tr>
<tr>
<td>HVD</td>
<td>hypervariable domain</td>
</tr>
<tr>
<td>JRAB</td>
<td>junctional Rab13-binding protein</td>
</tr>
<tr>
<td>LDLR</td>
<td>low-density lipoprotein receptor</td>
</tr>
<tr>
<td>LPH</td>
<td>lactase-phorizin hydrolase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MILCAL-L2</td>
<td>molecule interacting with CasL-like 2</td>
</tr>
<tr>
<td>MPR</td>
<td>mannose 6-phosphate receptor</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>PDEδ</td>
<td>phosphodiesterase δ subunit</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PKD</td>
<td>protein kinase D</td>
</tr>
<tr>
<td>RE</td>
<td>recycling endosomes</td>
</tr>
<tr>
<td>REP</td>
<td>Rab escort protein</td>
</tr>
<tr>
<td>RGGTase</td>
<td>Rab geranylgeranyl transferase</td>
</tr>
<tr>
<td>RP2</td>
<td>retinitis pigmentosa gene 2</td>
</tr>
<tr>
<td>RP3</td>
<td>retinitis pigmentosa gene 3</td>
</tr>
<tr>
<td>RPGR</td>
<td>retinitis pigmentosa GTPase regulator</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SNAP</td>
<td>synaptosomal-associated protein</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptors</td>
</tr>
<tr>
<td>SWI</td>
<td>switch region I</td>
</tr>
<tr>
<td>SWII</td>
<td>switch region II</td>
</tr>
<tr>
<td>TDO</td>
<td>triple dropout plates</td>
</tr>
<tr>
<td>TfnR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TGN</td>
<td><em>trans</em>-Golgi network</td>
</tr>
<tr>
<td>t-SNARE</td>
<td>target-SNARE</td>
</tr>
<tr>
<td>VAMP</td>
<td>vesicle-associated membrane protein</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>v-SNARE</td>
<td>vesicle-SNARE</td>
</tr>
<tr>
<td>VSVG</td>
<td>vesicular stomatitis virus glycoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>XLPR</td>
<td>retinitis pigmentosa with an X-linked hereditary pattern</td>
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CHAPTER 1: GENERAL INTRODUCTION
Polarized Epithelial Cells and Membrane Trafficking

Many cells are polarized which means they are asymmetrical in cell shape, function, and protein localization. Cell polarity is found in single-cell organisms, including yeast and bacteria, and in cells of multicellular invertebrates like Caenorhabditis elegans, Drosophila, and vertebrate (mammalian) tissues. There are diverse types of polarized mammalian cells, from neurons, which are polarized attenuated cells measuring up to several meters long, to polarized epithelial cells that are short rectangular shapes approximately 10 µm tall. In complex animals, most internal organ systems are lined by a monolayer of polarized epithelial cells. The epithelial plasma membrane has two functionally and biochemically distinct plasma membrane domains which are separated by tight junctions (Nelson, 2003). The apical domain is enriched in glycolipids and cholesterol and comprises the luminal wall of organs. The basolateral membrane comes in contact with connective tissue and is the functional home of multiple lipids, proteins and receptor proteins, including low-density lipoprotein receptor (LDLR). In order to maintain proper cell polarity, cells must continually sort transmembrane proteins to the appropriate apical or basolateral membrane during biosynthetic or endocytic delivery. Since the late 1970’s, the Madin-Darby canine kidney (MDCK) cells have been used as a model to study polarized protein targeting in epithelial cells. The molecular mechanisms behind protein trafficking have been extensively researched since it was shown that the influenza virus assembles at the apical membrane and the vesicular stomatitis virus assembles at the basolateral membrane. The virus assembly site is a cellular response to the membrane trafficking of their envelope glycoproteins, influenza virus hemagglutinin (HA) and vesicular stomatitis virus glycoprotein (VSVG) (Rodriguez-Boulan et al., 2005). Elucidation of trafficking routes and sorting mechanisms has
continued, but there is still much that is unknown. Figure 1-1 is a schematic of our current understanding of common biosynthetic cargo protein trafficking pathways in polarized epithelial cells (Fölsch, 2008)

In eukaryotic cells, proteins and lipids are transported between membrane-bound organelles by vesicles in the endocytic and secretory pathways. Vesicles bud from a donor compartment by a process that allows selective incorporation of cargo into the forming vesicles. The vesicles are then transported to a specific acceptor compartment where they unload their cargo upon fusion with the target membrane (Rodriguez-Boulan et al., 2005). There are three classes of protein-coated vesicles. Coatamer protein complex-I (COPI) and coatamer protein complex-II (COPII) coated vesicles mediate transport between the endoplasmic reticulum (ER) and the Golgi as well as within the Golgi itself (Antonny et al., 2005; Gurkan et al., 2006). Clathrin-coated vesicles (CCVs) are responsible for transport of proteins and lipids from the plasma membrane to endosomes and for transport out of the trans-Golgi network (TGN) (Bonifacino and Glick, 2004). During vesicle formation cytosolic clathrin triskelions assemble into a coat. Clathrin also interacts with a number of other proteins including the clathrin adaptor protein (AP) complexes. The adaptor proteins are responsible for recruiting clathrin to the membrane, selecting the vesicle cargo, and recruiting accessory proteins that regulate vesicle formation (Robinson, 2004).
Figure 1-1: Common membrane trafficking routes in polarized epithelial cells.

(1) direct sorting to the basolateral membrane from the TGN; (2) raft-dependent and raft-independent trafficking directly to the apical membrane; (3) sorting from the TGN to the recycling endosomes; (3a) sorting to the apical membrane via the recycling endosomes; and (3b) AP-1B-dependent sorting to the basolateral membrane via the recycling endosomes. Abbreviations: TGN: trans-Golgi network, RE: recycling endosomes (adapted from Fölsch, 2008)
Apical Sorting

Protein sorting is governed by the signals encoded in the cargo proteins themselves and is facilitated by cytosolic machinery that recognizes these signals (Rodriguez-Boulan et al., 2004). Generally, apical cargo proteins partition into glycolipid membrane rafts that facilitate delivery to the surface either through lipid-lipid or lipid-protein interactions (Schuck and Simons, 2004). Often in apical sorting N- and O-linked glycans are attached to the ectodomains of proteins. Although, N- and O-linked glycans usually function recessively relative to basolateral sorting signals, the removal of these glycan chains results in non-polarized release of luminal secretory proteins in MDCK cells (Alfalah et al., 1999; Scheiffele et al., 1995). Another common class of apical targeting determinants is the glycosylphosphatidylinositol (GPI) anchors, which direct the modified proteins to the membrane. Other apical sorting signals are encrypted in the ectodomain, transmembrane, or cytoplasmic domains of the protein.

The influenza HA protein contains apical sorting sequences in the transmembrane domain, which direct the incorporation of the protein into lipid rafts. The lipid-raft hypothesis suggests that proteins are sorted apically because they have an affinity for microdomains of glycosphingolipids and cholesterol that are assembled at the Golgi complex. The lipid rafts and their associated proteins form sorting platforms that upon recognition by special machinery are incorporated into apical transport intermediates at the TGN, recycling endosomes, or plasma membrane that deliver them to the apical plasma membrane (Rodriguez-Boulan et al., 2005).

In addition to enrichment in glycolipids rafts, adaptor proteins for apical delivery have also been described. Recently, FAPP2, a phosphatidyl-4-phosphate (PI[4]P) binding protein, was found involved in Rab-dependent delivery. FAPP2 has a Pleckstrin homology (PH) domain that binds PI(4)P and activated Arf1 at the TGN (Godi et al., 2004). FAPP2 knock down
experiments in MDCK cells result in kinetic delay of apical delivery of different cargos, including HA and GPI-anchored proteins (Vieira et al., 2005). Raft-dependent apical transport is well established, however, less is known about targeting of apical proteins that do not associate with lipid rafts such as lactase-phorizin hydrolase (LPH), gp114, and neurotrophin receptor, p75. Galectin-3 was shown to be associated with the luminal site of LPH-containing vesicles that were non-raft dependent and galectin-3 knock down inhibited apical delivery of LPH, gp114 and p75 (Delacour et al., 2006).

**Basolateral sorting and adaptor proteins**

Unlike apical targeting information, basolateral sorting information is most often encoded in the cytoplasmic tails of transmembrane proteins as small peptide motifs, either tyrosine or di-leucine-based. These sorting signals are *cis*-dominant over apical sorting information and are recognized by cytosolic adaptor proteins (Fölsch, 2005). Typically, these linear peptide signals are recognized by cytosolic complexes of the clathrin adaptor protein family, which interact with clathrin (Nakatsu and Ohno, 2003). There are four major classes (AP-1 through AP-4), each comprised of two large subunits, (α, γ, ε, δ and β1-ß4), a small subunit (σ1-σ4), and a medium subunit (µ1-µ4). The tyrosine-based sorting signals interact with the medium subunit, whereas the di-leucine-based signals may be recognized by the γ/σ1 subunits of AP-1 or δ/σ3 of AP-3 (Fölsch, 2005; Janvier et al., 2003). Epithelial cells express two AP-1 complexes, AP-1A and AP-1B. AP-1A mediates sorting of transmembrane proteins between the TGN and endosomes (Owen et al., 2004). It is thought upon recruitment of AP-1A to the TGN by activated Arf1, the medium subunit of AP-1A, µ1A is phosphorylated leading to a conformational change that
enables μ1A to bind cargo proteins. Once the vesicle is released from the donor membrane, μ1A is dephosphorylated to facilitate vesicle uncoating (Ghosh and Kornfeld, 2003). Polarized epithelial cells express a cell-specific adaptor complex, AP-1B that differs from AP-1A only in the incorporation of the medium subunits μ1B or μ1A, respectively. In contrast to AP-1A’s function at the TGN, AP-1B facilitates sorting from the recycling endosomes to the basolateral membrane in cooperation with Rab8. It is believed that AP-1B is regulated by phosphorylation of μ1B similar to the model suggested for AP-1A (Fölsch, 2005).

In contrast to AP-1A and AP-1B, AP-2 is involved in endocytosis and localizes to endocytic CCVs that are pinched off from the plasma membrane and fuse with the early endosomes (Owen et al., 2004). More over, AP-3 is predominantly located on endosomes and the TGN and is involved in endosomal and lysosomal transport of transmembrane proteins (Nakatsu and Ohno, 2003). Finally, AP-4 is localized to the TGN and is implicated in the basolateral sorting of LDLR and 46 kDa cation-dependent mannose 6-phosphate receptor (MPR46) in MDCK cells (Simmen et al., 2002).

Another class of adaptors that recognizes the short sequence motifs in the cytoplasmic tails of protein cargo are the Golgi-localized, γ-ear-containing, Arf-binding proteins (GGAs). The GGAs are a family of ubiquitously expressed, monomeric, motif-binding cargo/clathrin adaptors discovered by several groups in 2000. There are three GGAs (GGA1, GGA2, and GGA3) found in mammalian cells. These monomeric proteins are only 70 kDa compared to the 300 kDa heterotetrameric APs. Despite the difference in size, both families appear to carry out their functions in similar ways by binding cargo motifs, membrane components, clathrin, and other proteins involved in CCV formation. There are still other adaptor proteins involved in vesicular transport, some regulated by ubiquitination, such as the Epsins, and more that are cargo...
specific (Owen et al., 2004). One example of newly discovered adaptors are the Dab2 (disabled-2) and ARH (autosomal recessive hypercholesterolaemia) proteins, clathrin adaptors involved in the transport of LDLR (Maurer and Cooper, 2006).

Vesicular Transport

Membrane trafficking events must be tightly regulated at every step of transport. This includes vesicle formation as well as the fission of vesicles, transport, tethering, and subsequent fusion to the acceptor membrane (Fig. 1-2) (Bonifacino and Glick, 2004). Fission of the nascent vesicle should only occur once the proper cargo has been selected. One protein suggested to regulate this step at the TGN is the serine/threonine protein kinase D (PKD). In polarized epithelial cells PKD is required for transport of selected basolateral cargo (Yeaman et al., 2004). Bossard et al. provided further evidence that PKD has a function in membrane fission. Using siRNA constructs, PKD depletion inhibited trafficking of VSVG to the surface of HeLa cells. Expression of the dominant active PKD converted the TGN into small vesicles (Bossard et al., 2007).

Fission of vesicular transporters was most commonly thought to be mediated by the GTPase, dynamin. More recently, Bonazzi et al., reported that the carboxy-terminal binding protein 3/brefeldin A-ribosylated substrate (CtBP3/BARS) controls fission in basolateral transport from the TGN to the plasma membrane and in endocytosis, independent of dynamin (Bonazzi et al., 2005).

Once formed, the vesicles must travel along elements of the cytoskeleton to their proper final destination. It is believed that clustered lipid rafts recruit microtubule motors (such as
Figure 1-2: Steps of vesicle budding and fusion.

1. Coat assembly initiation. The membrane-proximal coat components in blue are recruited to the donor compartment by binding to membrane-specific GTPases in red and/or to a specific phosphoinositide. Transmembrane cargo proteins and SNAREs begin to gather at the assembling coat.

2. Budding. The membrane-distal components in green are added and polymerize into lattice structures. Membrane curvature increases.

3. Scission. The nascent vesicle is severed at the neck through accessory proteins.

4. Uncoating. Various events induce the loss of the coat including: inactivation of the GTPase, phosphoinositide hydrolysis, and uncoating enzymes. Cytosolic coat proteins are recycled.

5. Tethering. The uncoated vesicle moves to the acceptor membrane, possibly with guidance from the cytoskeleton, and is tethered to the acceptor membrane by the combination of a GTP-bound Rab and a tethering factor.

6. Docking. The v-SNARE and t-SNAREs assemble into a four-helix bundle.

7. Fusion. The SNARE complex promotes fusion of the vesicle and membrane lipid bilayers. Cargo is transferred to the acceptor membrane and the SNAREs are recycled (Bonifacino and Glick, 2004).
kinesin or dynein) directly or through adaptors. These motors generate tubular elements that move along the microtubule and actin cytoskeletons. Microtubules and actin-based motors (myosins I, II, Vb and VI) most likely participate in several stages of endocytic and biosynthetic trafficking (Rodriguez-Boulan et al., 2005). Myosin IIα has been implicated in basolateral secretion of transmembrane cargo (Tuxworth and Titus, 2000) and Myosin VI was specifically shown to be a crucial component in AP-1B-dependent, biosynthetic cargo sorting. More specifically, a functional complex of myosin VI, optineurin, and GTPase Rab8 play a role in basolateral membrane delivery of cargo (Au et al., 2007). Actin filaments may also play a role in apical recycling as tracks for myosin-driven movement of vesicles with Rab proteins, as reported for myosin VI and Rab11 (Hales et al., 2001). Evidence supports the role of microtubule motors in apical transport. Dominant negative tail constructs of the motor protein kinesin KIF5B blocked the transport of p75NTR to the apical membrane (Jaulin et al., 2007; Rodriguez-Boulan et al., 2005). A minus-end-directed microtubule motor, dynein, interacts with the C-terminus of rhodopsin, the light-transducing visual pigment of rod cells, and is crucially involved in the apical transport of rhodopsin in MDCK cells (Tai et al., 1999). Finally, apical membrane bound vesicles containing HA and annexin XIIIb domains were found associated with the negative-end directed microtubule motor, Kif3C (Noda et al., 2001). Delivery of transport vesicles to the distinct acceptor membranes has a high degree of fidelity. It is interesting to note that although the involvement of microtubule and actin cytoskeletons in vesicle transport has been reported; fidelity of vesicle transport to the apical or basolateral membrane remains high despite a disruption of the cytoskeleton (Grindstaff et al., 1998). One possibility is that the ultimate specifications of docking and fusion reside in the interactions between the transport vesicle and target membrane domain (Nelson, 2003).
Uncoated vesicles move to the acceptor membrane with possible guidance from the cytoskeleton, and then are tethered to the acceptor membrane by the combination of a GTP-bound Rab and a tethering factor. Annexins are shown to self-aggregate and localize to the TGN, endosomes, and apical membrane. And annexins may serve as tethering factors between apical carriers and the apical membrane (Gerke et al., 2005).

Vesicles in transport must fuse with the target membrane and soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) are an important part of this process. The SNARE hypothesis was proposed in 1994, stating that each type of vesicle carries a specific vesicle-SNARE (v-SNARE) that binds to a cognate target-SNARE (t-SNARE) on the target membrane (Rothman and Warren, 1994; Sogaard et al., 1994). Vesicles destined for the basolateral membrane have been shown to involve the exocyst, an eight-subunit complex, thought to facilitate tethering of basolateral vesicles with the target site (Yeaman et al., 1999). At least two of the exocyst subunits associate with AP-1B vesicles (Fölsch et al., 2003). After tethering, the complex may rearrange to bring the vesicle close to the membrane to allow for SNARE pairing and subsequent fusion (Munson and Novick, 2006). Plasma membrane SNAREs of the syntaxin family and the synaptosomal-associated protein of 23 kDa (SNAP-23) comprise the t-SNAREs. Upon arrival, fusion is promoted by the formation of a SNARE four-helix bundle with the corresponding vesicle-associated membrane proteins, VAMPs/v-SNAREs. Syntaxin 3 localizes to the apical membrane and forms SNARE pairs with the v-SNAREs, TIVAMP and SNAP-23 (Galli et al., 1998; ter Beest et al., 2005). Syntaxin 4 localizes to the basolateral membrane and was recently found to form SNARE pairs with the v-SNARE cellubrevin during exocytosis at the basolateral membrane and for fusion at the basolateral membrane (Fields et al., 2007).
The *trans*-Golgi network and recycling endosomes

Transmembrane proteins destined for the plasma membrane are synthesized on ribosomes and directly inserted into ER membranes. They then travel from the ER into the Golgi apparatus where final glycosylation occurs. Up to this point, apical, basolateral, and cargo destined for the lysosomes still travel together. At the TGN, cargo becomes segregated to be sorted according to their final destinations. The TGN is a dynamic network of tubular reticular membranes at the crossroads of the endocytic and exocytic pathways. The TGN has the ability to generate pleiomorphic carriers with multiple destinations including: the apical or basolateral plasma membrane, early or late endosomes, recycling endosomes, the Golgi stack, and other specialized compartments in the cell (De Matteis and Luini, 2008). As mentioned earlier the TGN serves as a platform for lipid rafts in apical sorting. Recently the lipid raft model was refined to include the fact that a subdomain in the TGN requires both segregation in lipid domains and aggregation to permit extrusion from the TGN into a specific lipid raft transport carrier (Bard and Malhotra, 2006). Influenza HA sorts to the apical membrane from the TGN, and its apical sorting signal is encoded in its transmembrane domain that associates with lipid rafts (Scheiffele et al., 1997).

Less is known about the ‘direct’ transport of newly synthesized cargo from the TGN to the basolateral membrane. The direct pathway is considered one that does not include the recycling endosomes during biosynthetic delivery. One cargo sorted along this direct pathway during biosynthetic delivery is the mouse macrophage Fc receptor, FcII-B2 (FcR) (Fields et al., 2007). FcR is found in a variety of cells as the receptor for the Fc fragment of immunoglobulins. For example, it recognizes immunoglobulins, IgG and IgE (Matter and Mellman, 1994). FcR contains a di-luecine sorting signal and is sorted from the TGN to the basolateral membrane with an unknown adaptor (Fields et al., 2007).
Another cargo that travels along the direct path to the plasma membrane is the LDLR mutant, LDLR(Y18A) (Fields et al., 2007). The LDLR cytoplasmic tail contains two basolateral sorting determinants (Matter et al., 1992). The proximal signal located closer to the transmembrane domain is a FXNPXY motif (tail residues 13-18) and is recognized by the recycling endosomes (Fields et al., 2007). The distal targeting determinant is considered a non-canonical tyrosine-based motif, GYSY and is AP-1B-independent (Fields et al., 2007). In sorting of LDLR(Y18A), the GYSY sorting motif is the active basolateral sorting signal interacting with adaptor proteins in transport and it is sorted from the TGN directly to the basolateral membrane perhaps involving AP-4 (Fields et al., 2007). Transferrin receptor (TfnR) is also sorted from the TGN directly to the basolateral membrane during biosynthetic delivery before endocytic delivery to recycling endosomes (Gravotta et al., 2007; Sheff et al., 1999). The sorting signals in both LDLR(Y18A) and TfnR interact with μ4/AP-4. Antisense RNA expression against μ4 resulted in mild sorting defects for LDLR in MDCK cells (Simmen et al., 2002). Although ‘direct’ sorting refers to a pathway that does not involve the recycling endosomes, it is unclear if this direct pathway involves basolateral early endosomes (Fölsch, 2008).

In recent years, the recycling endosomes have become appreciated as bonafide sorting stations of newly synthesized cargo. Therefore recycling endosomes sort biosynthetic cargo transported from the TGN and endocytic cargo delivered from the plasma membrane (Ang et al., 2004; Cancino et al., 2007; Fölsch, 2005). For example, recycling endosomes serve as the sorting center for newly synthesized VSVG and A-VSVG to the basolateral and apical membranes, respectively. Ang et al. found by video microscopy, immunoelectron microscopy, and cell fractionation that the cargo VSVG entered transferrin-positive recycling endosomes
minutes after exit from the TGN. They also showed that enzymatic inactivation of recycling endosomes blocked VSVG delivery to the cell surface. The apically targeted mutant (A-VSVG) behaved similarly (Ang et al., 2004). In addition, others demonstrated that the polymeric immunoglobulin receptor (pIgR) travels through endosomes before reaching the apical surface (Orzech et al., 2000). As mentioned earlier, the proximal targeting determinant of LDLR is a FXNPXY tyrosine-based motif and the truncated version of LDLR (LDLR-CT27) is sorted to the basolateral membrane via the recycling endosome. VSVG and LDLR-CT27 are examples of cargos dependent on AP-1B at the recycling endosomes, during biosynthetic delivery to the basolateral membrane (Ang et al., 2004; Fields et al., 2007). We have a much better understanding of transport of cargo from the recycling endosomes to the basolateral membrane, while still very little is known about how the trafficking between the TGN and recycling endosomes is regulated. Also noted earlier, PKD is involved in the fission of carriers originating from the TGN. Overexpression of kinase dead PKD leads to apical missorting of VSVG (Bossard et al., 2007; Yeaman et al., 2004).

Rab Proteins: Structure and Function in Vesicular Transport

Timing in the generation of transport vesicles, movement, and subsequent fusion to the acceptor membrane is critical to successful protein transport. Polarized secretion of transport vesicles involves the Ras superfamily of GTPases, a large family of proteins that bind guanosine-5'-triphosphate (GTP) or guanosine diphosphate (GDP). GTPases act like molecular switches in many cellular processes including growth, motility and protein trafficking. This switching mechanism imposes temporal and spatial regulation on GTPase-dependent events. The small
Ras-related GTP-binding proteins constitute a superfamily which can be grouped into four main branches according to their sequence similarities: Ras, Rho/Rac, Rab/Ypt/Sec4 and Arf. The largest branch of the Ras superfamily is the Rab/Ypt/Sec4 family, which regulates vesicular transport pathways. In mammalian cells, over 60 different Rab proteins have been identified. A large number of studies have contributed to the knowledge that Rab proteins are distributed to distinct intracellular compartments and regulate transport between organelles (Munro, 2002; Pfeffer and Aivazian, 2004; Pfeffer, 2001; Zerial and McBride, 2001). Figure 1-3 summarizes some of the intracellular localizations of Rab proteins in mammalian cells (Zerial and McBride, 2001).

Rab proteins undergo a cycle of GTP hydrolysis and nucleotide exchange that regulates their activation state and is also coordinated with rounds of membrane association and dissociation. Only GTP-bound Rab proteins are stably associated with membranes, whereas GDP-bound Rab proteins are extracted from membranes by GDP-dissociation inhibitor (GDI). In addition, the nucleotide state is actively regulated through guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). A GEF stimulates replacement of GDP with GTP. In its active GTP-bound state, Rab proteins can interact with effector molecules to regulate cellular functions. After membrane docking and fusion, a GAP stimulates the GTPase activity of the Rab protein, leading to hydrolysis and dissociation of the Rab from its effectors. Then the GDI extracts Rab-GDP from the membrane and recycles it to the donor membrane for another round of transport (Grosshans et al., 2006; Zerial and McBride, 2001). For example, in yeast, polarized exocytic transport to the emerging bud is regulated by Sec4p, which is activated by the exchange factor Sec2p. Sec4p-GTP can then bind Sec15p, a subunit of the exocyst complex, a vesicle-tethering complex important for exocytosis (Guo et al., 1999). The GTP- and
Figure 1-3: Intracellular localization of Rab proteins.
GDP-bound states impose temporal and spatial regulation to membrane transport. The Rab proteins’ on/off regulatory functions are restricted to the membrane compartments where they are localized and are regulated by the intrinsic and catalyzed rates of nucleotide exchange and hydrolysis. The very specific localization of Rab proteins within the cell has been used as identification markers for cellular organelles and their subdomains (Ali and Seabra, 2005; Grosshans et al., 2006; Zerial and McBride, 2001).

Rab proteins are extremely well conserved over evolution. In a few cases, yeast and mammalian Rab proteins are functionally interchangeable. Ypt51p is the mammalian homolog of Rab5 and both are involved in early endocytosis. Ypt51p expression in animal cells localizes to Rab5-positive early endosomes and also stimulates endocytosis (Singer-Kruger et al., 1995). Rab1a can complement loss of Ypt1 in Saccharomyces cerevisiae with both proteins regulating transport between the ER and Golgi complex (Haubruck et al., 1989). Rab proteins have additional regions of sequence homology with Sec4 and Ypt1 that are not present in other members of the Ras-family, Ras and Rho GTPases.

The domain structure of a Rab protein is presented in Figure 1-4 (Ali and Seabra, 2005). A Rab protein contains a C-terminal prenylation motif, a hypervariable domain, and two switch regions (SWI and SWII). The prenylation motif is needed for the addition of geranylgeranyl groups responsible for attachment to membranes. The switch regions are flexible loop domains that change conformation upon the exchange of GDP for GTP. An additional characteristic of Rab proteins is their N-terminal extensions that could form small flexible domains that may be involved in interactions with specific target proteins (Pereira-Leal and Seabra, 2000; Pereira-Leal and Seabra, 2001). In a large database analysis of the mammalian Rab family, five Rab-conserved sequences were designated the Rab family motifs (RabF). These RabF regions were
Figure 1-4: The domain structure of a generic Rab protein.

(A) A schematic representation of the domain structure of a Rab protein. The RabF (red) and RabSF (yellow) regions as identified by Pereira-Leal and Seabra (Pereira-Leal and Seabra, 2001). The black bars indicate the locations of the switch regions (SWI and SWII) and the hypervariable domain (HVD). The GG prenylation motif is located at the C-terminal end of the protein. (B) A three-dimensional model of Rab5a with motifs colored as in A. Only a portion of the hypervariable domain is shown due to truncation (Zhu et al., 2004).
found to cluster in and around the switch I and switch II regions. Rab subfamily (RabSF) regions were also defined as depicted in Figure 1-4 (Pereira-Leal and Seabra, 2000). Rab proteins are synthesized in the cytosol as soluble proteins that are post-translationally modified by prenylation at their C-terminus by two geranylgeranyl groups, in most cases (Leung et al., 2003).

Protein prenylation involves the covalent addition of a geranylgeranyl (20-carbon) isoprenoid onto C-terminal cysteines through thioester bonds. The newly synthesized Rab proteins are first recognized by a soluble chaperone-like protein Rab escort protein (REP). Next, the REP presents the Rab protein to Rab geranylgeranyl transferase (RGGTase) for the addition of the geranylgeranyl group(s). These lipid moieties allow the attachment of the Rab protein to the lipid bilayer of the organelle (Ali and Seabra, 2005). Prenylation occurs at the C-terminal cysteine motifs. Rab proteins usually undergo double geranylgeranylation within CC, CXC, or CCXX motifs, and both cysteines are modified by geranylgeranyl lipid groups. Unlike the other Rab proteins, Rab8, Rab11, and Rab13 terminate with a CaaX motif similar to those of the Ras/Rho proteins in which C is a cysteine, a is an aliphatic residue and X is any amino acid. In these cases the Rab proteins are isoprenylated (Joberty et al., 1993).

Rab13 family members

In mammalian cells among the closest homologues of Sec4p are Rab8, Rab10, and Rab13 (Pereira-Leal and Seabra, 2001). Both Rab8 and Rab13 are highly expressed in the kidney and lungs, whereas Rab10 seems to be less abundant in these tissues (Elferink et al., 1992). As mentioned, Rab8 plays a role in exocytosis of AP-1B-dependent cargo from the recycling
endosomes to the basolateral membrane. Ang et al. published findings in which expression of the constitutively active GTP hydrolysis mutant of Rab8 selectively resulted in the missorting of newly synthesized AP-1B-dependent cargo to the apical membrane in MDCK cells. This missorting phenotype was demonstrated with AP-1B-dependent cargos, VSVG and LDLR, but not for AP-1B-independent cargos, A-VSVG and FcR. Similar results were seen for the Cdc42 dominant negative mutant, previously implicated in basolateral sorting (Ang et al., 2003). Although Rab8 regulates basolateral delivery, it does not appear to be involved during recycling (Henry and Sheff, 2008). Again, Myosin VI was specifically shown to be a component in AP-1B-dependent cargo sorting and the functional complex of myosin VI, optineurin, and Rab8 appears to play a role in basolateral membrane delivery (Au et al., 2007). In addition to the roles of Rab8 in basolateral sorting and actin-based movement, it was recently published that Rab8a activity is necessary for the outgrowth of the primary cilium (Nachury et al., 2007; Yoshimura et al., 2007). Rab8a knock out mice have polarity defects in intestinal cells including a shortening of the microvilli (Sato et al., 2007).

Less is known about the function of Rab10, although it is thought to regulate endosomal sorting of internalized cargo. Babbey et al. demonstrated Rab10 colocalizes with TfnR-positive recycling endosomes. The authors claim that through endocytic kinetic analyses of TfnR and IgA they see increased recycling from early compartments with expression of mutant forms of Rab10 (Babbey et al., 2006). Chen and colleagues identified Rab10 as a key regulator of endocytic recycling in the Caenorhabditis elegans intestine. Rab10 null mutant intestinal cells accumulate abnormally abundant and enlarged Rab5-positive early endosomes (Chen et al., 2006). Another study found Rab10 involved in basolateral transport in MDCK cells. In the presence of activated Rab10, VSVG was unable to leave the Golgi as monitored by
endoglycosidase H (endoH) resistance in coverslip-grown nonpolarized cells. Activated Rab10 expression also resulted in the missorting of VSVG from the basolateral to apical surface in polarized cells (Schuck et al., 2007).

Previously Rab13 has been shown to play a role in tight junction integrity. Marzesco and colleagues demonstrated that in MDCK cells stably expressing GFP-Rab13 mutants, the expression of the dominant active allele Rab13Q67L but not the GDP-locked, dominant negative form, Rab13T22N led to a delay of tight junction formation as monitored by transepithelial electrical resistance (Marzesco et al., 2002). Rab13 was found to regulate protein kinase A (PKA) signaling during tight junction assembly. Rab13Q67L directly bound to PKA and inhibited PKA-dependent phosphorylation and recruitment of the vasodilator-stimulated phosphoprotein (VASP) to the tight junctions (Kohler et al., 2004). Another group is investigating a role for Rab13 in mediating the continuous endocytic recycling of occludin to the tight junctions. Sasaki identified MILCAL-L2 (molecule interacting with CasL-like 2) as a novel Rab13-binding protein and renamed it JRAB (junctional Rab13-binding protein). JRAB specifically bound to the GTP-bound form of Rab13 and Rab8 at its C-terminus and JRAB mutants lacking the binding domain inhibited the endocytic recycling of occludin. Rab13 as well as JRAB mediate endocytic recycling of occludin but not TfnR (Morimoto et al., 2005; Terai et al., 2006).

The only protein known to regulate the GTPase cycle of Rab13 is the rod cGMP phosphodiesterase δ subunit (PDEδ) that acts as the Rab-GDI. As mentioned earlier, many Rab proteins form complexes with Rab-GDI in the cytosol. Rab-GDI has the ability to retrieve GDP-bound Rab proteins from membranes. PDEδ was found to interact with the isoprenylated form
of Rab13 and has the ability to dissociate Rab13 from cellular membranes (Marzesco et al., 1998).

The studies presented in this dissertation investigate mechanisms by which cells establish polarity. These investigations have led to the important discovery of a function for Rab13 in biosynthetic delivery of transmembrane proteins. Chapter 2 describes experiments demonstrating a role for Rab13 in vesicular transport of apical and basolateral cargo between the TGN and recycling endosomes. Chapter 3 examines the conservation of sorting signals across different cell types, specifically polarized neurons. The sorting of the axonal cell adhesion molecule L1/NgCAM has been studied in neuronal cells. This analysis supports the conservation of sorting signals and helps to elucidate the path of transcytosis observed in the biosynthetic transport of NgCAM to the apical membrane. The discussion in Chapter 4 attempts to synthesize all the lines of investigations pursued in this dissertation. In addition, the Appendix reports analysis of Rab13 yeast two-hybrid interactions as well as the results of other experiments in which transmembrane cargo proteins are co-expressed with Rab13 mutants in MDCK cells. Experiments described in this dissertation help to elucidate the underlying principles of how cells establish polarity, specifically in the sorting of transmembrane cargos.
CHAPTER 2: RAB13 REGULATES MEMBRANE TRAFFICKING BETWEEN THE TRANS-GOLGI NETWORK AND RECYCLING ENDOSOMES
**Introduction**

To maintain apical-basolateral polarity, polarized epithelial cells must continuously sort transmembrane proteins to their correct location during biosynthetic and endocytic delivery (Rodriguez-Boulan *et al.*, 2005). Sorting of internalized cargo takes place in perinuclear recycling endosomes, whereas sorting of newly synthesized cargo takes place in the trans-Golgi network (TGN) or in the recycling endosomes (Ang *et al.*, 2004; Cancino *et al.*, 2007; Fölsch, 2005). One cargo thought to follow a direct pathway from the TGN to the apical membrane is the influenza hemagglutinin (HA) protein (Fullekrug and Simons, 2004). Basolateral cargos most likely following a direct pathway from the TGN to the surface are the mouse macrophage Fc receptor, FcII-B2 (FcR) and the mutant low-density lipoprotein receptor (LDLR), LDLR(Y18A) (Fields *et al.*, 2007). HA is segregated into glycolipid rafts for transport to the apical membrane, while basolateral cargo such as FcR and LDLR(Y18A) may interact with adaptor proteins that are recruited to the TGN such as AP-4 (Fields *et al.*, 2007; Fullekrug and Simons, 2004). Examples of cargos moving from the TGN into recycling endosomes during biosynthetic delivery to the plasma membrane are vesicular stomatitis virus glycoprotein (VSVG), the apical variant, A-VSVG, and the truncated version of LDLR (LDLR-CT27) (Ang *et al.*, 2004; Fields *et al.*, 2007; Gravotta *et al.*, 2007). From the recycling endosome, cargos destined for the basolateral membrane frequently rely on the epithelial-specific adaptor complex AP-1B for sorting (Fields *et al.*, 2007; Fölsch, 2005). Cargo traveling to the apical membrane may segregate into Rab11-positive apical recycling endosomes before being delivered to the apical membrane (Casanova *et al.*, 1999). Other transmembrane proteins such as the TGN resident protein TGN38 travel through recycling endosomes after internalization from the plasma
membrane on the way back to the TGN (Ghosh et al., 1998). Despite our increasing knowledge of membrane trafficking pathways between the recycling endosomes and the plasma membrane, we know very little about the trafficking pathway from the TGN to the recycling endosomes.

Eukaryotic cells contain a variety of small 21-27 kDa guanine nucleotide-binding proteins. Although these proteins are structurally distinct from each other and from p21ras, they share significant homologies with p21ras, particularly in the domains involved in guanosine-5′-triphosphate (GTP)/guanosine diphosphate (GDP) binding and in GTP hydrolysis. Numerous members of the ras-related GTPase superfamily have been identified and classified according to their sequence similarities. Among them, the proteins of the Rab/Ypt/Sec4 family are key regulators in vesicular trafficking (Pereira-Leal and Seabra, 2000). For example, in yeast cells polarized exocytic transport to the emerging bud is regulated through sequential action of GTPases such as Cdc42, the Rab protein Sec4p, and RhoA (Fukata et al., 2003; Guo et al., 1999). Ypt1p is essential for endoplasmic reticulum (ER) to Golgi trafficking in yeast (Balch, 1990). Interestingly, many features of polarized secretion in yeast are also used for basolateral secretion in epithelial cells. For example, Cdc42 controls basolateral delivery of VSVG (Kroschewski et al., 1999). In mammalian cells the closest homologues of Sec4p are Rab8, Rab10, and Rab13 (Pereira-Leal and Seabra, 2001). Rab8 regulates exocytosis of AP-1B-dependent cargo from the recycling endosomes to the basolateral membrane (Ang et al., 2003). In addition, Rab8a is important for the outgrowth of the primary cilium (Nachury et al., 2007; Yoshimura et al., 2007), and overall polarity in intestinal cells (Sato et al., 2007). Rab10 regulates endosomal sorting of internalized cargos (Babbey et al., 2006), and/or surface delivery of newly synthesized basolateral cargos (Schuck et al., 2007).
In contrast to the functions suggested for Rab8 and Rab10, Rab13 plays a role in tight junction formation and integrity. In MDCK cells stably expressing the dominant active GFP-tagged Rab13Q67L allele mutants, the expression of the dominant active allele Rab13Q67L led to a delay of tight junction formation. This was monitored by transepithelial electrical resistance. The same effect was not observed with the dominant negative allele (Marzesco et al., 2002). This effect may be partially attributed to an impaired endocytic recycling of the tight junction proteins claudin-1 and occludin and a down regulation of PKA activity (Kohler et al., 2004; Marzesco et al., 2002; Morimoto et al., 2005; Yamamoto et al., 2003).

In this study, we investigated whether Rab13 might have additional functions in membrane trafficking. We found that in addition to the plasma membrane, Rab13 partially co-localizes with markers of the TGN (TGN38) and markers of the recycling endosomes (TfnR). In agreement with this localization, overexpression of either the dominant active mutant protein, Rab13Q67L, or the dominant negative mutant protein, Rab13T22N, resulted in severe defects in membrane delivery of newly synthesized proteins that travel through recycling endosomes on their way to the surface. In addition, overexpression of mutant Rab13 also interfered with localization of TGN38 at the TGN. Our results suggest, that Rab13’s primary role is regulating membrane trafficking events between the TGN and recycling endosomes.

Results

Overexpression of Rab13 mutant alleles by microinjection does not interfere with cell polarity

To analyze the role of Rab13 in membrane trafficking along the biosynthetic pathway, two different mutants were generated using site-directed mutagenesis. The so-called dominant
active mutant, Rab13Q67L, contains a glutamine to leucine substitution essentially locking the protein in active conformation bound to membranes. The corresponding mutation in other ras and rab small GTPases inhibits both the intrinsic and GTPase-activating protein (GAP)-stimulated GTPase activity and therefore is deficient in GTP hydrolysis. The mutant cannot undergo catalytic cycling to the GDP-bound form and remains bound to its effectors on the target membranes. Therefore, this mutant is considered an activated form of the GTPase (Der et al., 1986; Stenmark et al., 1994). The dominant negative mutant, Rab13T22N, contains a threonine to asparagines substitution in the first GTP/GDP-binding motif. In other ras and other rab proteins, this mutant has a lower affinity for GTP than for GDP and is expected to be inactive (Feig and Cooper, 1988; Stenmark et al., 1994). This mutant also appears to sequester guanine nucleotide exchange factors and thereby inhibits the function of the endogenous GTPase (Burstein et al., 1992). Furthermore, Rab13 mutants were tagged with the V5-epitope at the N-terminus of the protein.

The V5-tagged Rab13Q67L (dominant active allele) or V5-tagged Rab13T22N (dominant negative allele) cDNAs were microinjected into filter-grown, fully polarized, MDCK cells. First we tested whether overexpression of these mutants had any effects on the general polarity of the cell. As shown in Fig. 2-1 A, neither Rab13Q67L nor Rab13T22N overexpression disrupted basolateral localization of the marker glycoprotein (gp), gp58. Furthermore, tight junction assembly as judged by ZO-1 staining and cilium biogenesis were not affected in this assay (Fig. 2-1 B). Therefore, within short time points (4 h) of overexpression achieved by microinjection, the overall polarity is unaffected as measured by the tight junction and basolateral membrane markers.
Figure 2-1: Overexpression of Rab13 mutants by microinjection does not affect cell polarity as judged by gp58, ZO-1, and cilium staining.

Filter-grown MDCK cells were microinjected with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N. After microinjection cells were incubated at 39°C for 2 h followed by a 2 h chase at 31°C in the presence of CHX and processed for immunofluorescence staining. Rab13 protein was visualized with anti-V5 antibodies followed by fluorescently labeled secondary goat anti-mouse IgG2a antibodies. (A) Cells were co-stained for Rab13 (in red, Alexa 594) and gp58 (in green) with anti-gp58 antibodies and Alexa 488-labeled goat anti-mouse IgG1 antibodies. (B) Cells were triple-labeled for Rab13 (in blue, Cy5), acetylated tubulin to label primary cilia (in red, using anti-acetylated tubulin primary and 594-labeled goat anti-mouse IgG2b secondary antibodies), and ZO-1 (in green, using anti-ZO-1 primary and 488-labeled goat anti-mouse IgG1 secondary antibodies). (A, B) Specimens were analyzed by confocal microscopy and representative XZ images are shown.
Overexpression of Rab13 mutants inhibits surface arrival of VSVG

Using microinjection, we next tested the hypothesis that Rab13 mutants have adverse effects on surface delivery of VSVG. VSVG is a transmembrane protein that passes through the canonical biosynthetic pathway, from the ER and Golgi to the TGN. VSVG then traverses the recycling endosomes and is sorted to the basolateral membrane in the AP-1B pathway (pathways 3 and 3b in Fig. 2-5 A). Filter-grown MDCK cells were co-injected with cDNAs encoding either Rab13 mutants or a CFP-tagged, temperature-sensitive mutant of VSVG (VSVG-CFPts045) (Toomre et al., 1999). Cells were incubated for 2 h at the non-permissive temperature of 39°C at which VSVG accumulates in the ER while Rab13 mutants were produced in the cytosol. To release VSVG from the ER and allow for surface delivery, cells were then shifted to 31°C for 2 h in the presence of cycloheximide (CHX) to prevent further protein synthesis. In order to detect VSVG localized at the plasma membrane, cells were stained with an antibody recognizing the VSVG ectodomain prior to fixation. Subsequently cells were fixed, permeabilized and stained for Rab13 with anti-V5 antibodies and total VSVG with anti-GFP antibodies. Whereas VSVG arrived at the basolateral surface in control cells, co-injection of either mutant allele led to a severe block in surface delivery of VSVG (Fig. 2-2 A). We noted that the signal intensity of VSVG also decreased indicating perhaps a missorting into lysosomes. Therefore, we incubated the cells with lysosomal inhibitors (50 µM ammonium chloride) and proteasomal inhibitors (3 mM MG132) to inhibit protein degradation during the chase (when VSVG is released from the ER at 31°C). This treatment enhanced the signal for VSVG without restoring surface delivery (Fig. 2-2 B). Overall, we observed impaired surface delivery in 98% of cells analyzed.
Figure 2-2: Overexpression of Rab13 mutants disrupts surface delivery of VSVG.

Filter-grown MDCK cells were microinjected with cDNAs encoding VSVG-CFPts045 alone or together with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N. After microinjection cells were incubated at 39°C for 2 h followed by a 2 h chase at 31°C in the presence of CHX. Rab13 protein was visualized with anti-V5 antibodies followed by fluorescently labeled secondary goat anti-mouse IgG2a antibodies. Prior to fixation, cells were processed for surface staining with anti-VSVG antibodies (TK1), fixed and incubated with anti-GFP and anti-V5 antibodies followed by an incubation with fluorescently labeled secondary antibodies: goat anti-rabbit Alexa 488 (total VSVG-CFP), goat anti-mouse IgG1 Alexa 594 (surface VSVG), and goat anti-mouse IgG2a Cy5 (V5-Rab13).

(B) Experiment performed as described above with one exception. The 2 h chase at 31°C was performed in the presence of CHX, 0.05 mM MG132, and 3 mM ammonium chloride. (A, B) Specimens were analyzed by confocal microscopy and representative XZ images are shown.
expressing Rab13Q67L, and in 97% of cells expressing Rab13T22N. Control cells only failed to deliver VSVG to the surface in 5% of all cells analyzed (Fig. 2-3).

Overexpression of Rab13 mutants inhibits surface arrival of cargo proteins that move from the TGN into recycling endosomes during biosynthetic delivery

Since the ER is commonly a site where proteins fail to exit, we tested whether Rab13 functions before or after VSVG moved into the Golgi complex. To this end, coverslip-grown MDCK cells were co-injected with cDNAs encoding VSVG-CFPts045 and V5-tagged Rab13 mutants. Subsequently, cells were incubated at 39°C for 2 h followed by 2 h at 20°C in the presence of CHX. During the 20°C incubation, VSVG can exit the ER but becomes trapped in the Golgi. Specimens were fixed and immunolabeled for the cis-Golgi marker GM130, CFP and V5-Rab13. As shown in Fig. 2-4, there was no discernible difference in the transport of VSVG into the Golgi complex as judged by co-localization of VSVG and GM130 between control cells and those co-injected with Rab13 mutant alleles. This co-localization was observed in virtually 100% of cells analyzed independent of Rab13 overexpression. Thus, Rab13 may function in surface delivery of proteins downstream of the Golgi complex.

To narrow down which post-Golgi trafficking steps might be affected, we tested whether overexpression of Rab13 mutants by microinjection would affect the surface delivery of other reporter proteins as well. First we analyzed the LDL receptor truncation mutant LDLR-CT27. This receptor is sorted to the basolateral membrane in an AP-1B-dependent pathway similar to
Figure 2-3: Quantification of surface delivery. Overexpression of Rab13 mutants disrupts surface delivery of VSVG, A-VSVG, and LDLR-CT27.

Mock-injected control cells (M), or cells co-injected with cDNAs encoding V5-Rab13Q67L (Q) or V5-Rab13T22N (T) together with cDNAs encoding VSVG, A-VSVG, or LDLR-CT27 were scored for surface delivery (blue bars) or impaired surface delivery (red bars) of the reporter protein. Data collected for VSVG and A-VSVG represent mean values from at least four independent experiments counting between 55 and 137 individual cells per experimental condition treated with or without proteolysis inhibitors together to enhance the statistical value (note there was no statistical difference in surface delivery between cells treated with or without proteolysis inhibitors). Data collected for LDLR-CT27 represent mean values from three independent experiments counting 34 cells (control), 29 cells (Rab13Q67L), or 18 cells (Rab13T22N). Error bars indicate SD.
Figure 2-4: Overexpression of Rab13 mutants has no affect on VSVG transport to the Golgi complex.

MDCK cells seeded on coverslips were co-injected with cDNAs encoding VSVG-CFPts045 alone (upper panel) or together with cDNAs encoding V5-Rab13Q67L (middle panel) or V5-Rab12T22N (bottom panel). Post-injection, cells were incubated for 2 h at 39°C followed by a chase at 20°C for 2 h in the presence of CHX. Subsequently, cells were fixed and immunolabeled for CFP (anti-GFP antibodies), Rab13 (anti-V5 antibodies), and GM130 (610822 antibodies) followed by an incubation with the following secondary antibodies: goat anti-rabbit Alexa 488 (CFP), goat anti-mouse IgG2a Cy5 (V5-Rab13), and goat anti-mouse IgG1 Alexa 594 (GM130). For quantification, mock-injected cells or cells co-injected with VSVG and Rab13 mutants were scored for co-localization between VSVG and GM130. Values given in the text represent mean values from at least three independent experiments examining a total number of at least 30 cells per condition. Specimens were analyzed by confocal microscopy and representative XY images are shown. Scale bars are 10 μm.
VSVG (pathways 3 and 3b in Fig. 2-5 A) (Fields et al., 2007). We found that like VSVG, the surface delivery of LDLR-CT27 was severely inhibited by both Rab13Q67L (93% of cells analyzed) and Rab13T22N (90% of cells analyzed) overexpression (Fig. 2-3 and Fig. 2-5 B).

The next two reporter proteins tested were the LDLR mutant LDLR(Y18A), which has an intact GYSY sorting motif and mouse macrophage Fc receptor (FcR) that has a LL-based sorting motif for basolateral delivery (Matter and Mellman, 1994). Both receptors are thought to travel directly from the TGN to the basolateral surface independent of AP-1B function (pathway 1 in Fig. 2-5 A) (Fields et al., 2007; Roush et al., 1998). As shown in Figs. 2-5 C and D, the overexpression of Rab13 mutants had no effect on surface delivery of LDLR(Y18A) or FcR.

We next tested the transport of the influenza HA protein when co-injected with the Rab13 mutants. It is believed that the HA protein is sorted directly from the TGN to the apical membrane (pathway 2 in Fig. 2-5 A) (Schuck and Simons, 2004). We found no inhibition of surface delivery for HA with the co-expression of the Rab13 mutants (Fig. 2-5 E).

Another cargo, apical VSVG (A-VSVG) moves through recycling endosomes on its way to the apical surface (pathway 3 and 3a in Fig. 2-5 A) (Ang et al., 2004). In contrast to the delivery of the apical cargo HA, we observed a severe inhibition of surface delivery for A-VSVG upon co-expression of Rab13Q67L or Rab13T22N (Fig. 2-6 A). Again, we found decreased fluorescent signals of A-VSVG in the cells. However, as was the case for VSVG, inhibition of protein degradation with ammonium chloride (50 µM) and MG132 (3 mM) raised the signal intensity without rescuing surface delivery (Fig. 2-6 B). The lack of surface arrival for A-VSVG was observed in 100% of cells analyzed expressing Rab13Q67L and in 100% of cells analyzed expressing Rab13T22N, while 98% of mock injected cells delivered A-VSVG to the surface (Fig. 2-3).
Figure 2-5: Rab13 overexpression affects selective cargos.

(A) Model that depicts the most common trafficking pathways between the TGN and either apical or basolateral plasma membrane (see text for details).  (B-E) Fully polarized MDCK cells were co-injected with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N and cDNAs encoding various reporter proteins. Following surface staining for the reporter proteins, permeabilized cells were stained for V5-Rab13 with anti-V5 antibodies and fluorescently labeled anti-mouse IgG2a antibodies (Cy5-labeled in B and C, and Alexa 488-labeled in D and E).  (B-C) cDNAs encoding V5-Rab13 mutants were co-injected with cDNAs encoding either LDLR-CT27 (B) or LDLR(Y18A) (C).  Injected cells were incubated for 1 h at 37˚C followed by 4 h at 20˚C and a 2 h chase at 37˚C in the presence of CHX.  Surface staining was achieved with anti-LDLR antibodies (C7) followed by Alexa 594-labeled secondary goat anti-mouse IgG2b antibodies.  (D) For expression of FcR, cDNAs encoding mRFP-tagged FcR were co-injected with cDNAs encoding Rab13 mutants.  Post-injection, cells were incubated for 1 h at 37˚C and 2 h at 20˚C followed by a 2 h chase at 37˚C in the presence of CHX.  For surface staining, cells were incubated with anti-FcR antibodies (2.4G2) followed by Cy5-labeled secondary goat anti-rat antibodies.  (E) Cells microinjected to express Rab13 mutants together with HA were incubated for 1 h at 37˚C followed by 4 h at 20˚C and a 2 h chase at 37˚C in the presence of CHX.  Surface staining was achieved by applying a goat anti-HA ectodomain antibody followed by Alexa 594-labeled secondary anti-goat antibodies.  (B-D) Specimens were analyzed by confocal microscopy and representative XZ images are shown.
Figure 2-6: Overexpression of Rab13 mutants disrupts surface delivery of A-VSVG.

Filter-grown MDCK cells were microinjected with cDNAs encoding A-VSVG-CFPts045 alone or together with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N. Experimental protocol is as described in Figure 2-2. Specimens were analyzed by confocal microscopy and representative XZ images are shown.
Rab13 localizes to the TGN and the recycling endosomes

Rab13 was previously localized to the basolateral plasma membrane in addition to intracellular structures (Marzesco et al., 2002). Our data indicate that Rab13 has an additional function at the TGN or recycling endosomes; therefore, we sought to determine the intracellular location of Rab13 in more detail. To this end, we generated defective adenoviruses to express low levels of wild type GFP-tagged Rab13 (GFP-Rab13) in MDCK cells. The defective adenoviruses were used to infect MDCK cells grown on coverslips and analyze the localization of GFP-Rab13 relative to various marker proteins. First, we co-infected cells with defective adenoviruses encoding GFP-Rab13 or either µ1A-HA or µ1B-myc to monitor Rab13’s localization relative to AP-1A or AP-1B, respectively. Rab13 co-localized with AP-1A at the TGN in 97 +/- 7% of cells analyzed, with some Rab13 staining localized to different structures (Fig. 2-7 A). It should be noted that although Rab13 did not co-localize entirely with AP-1A, almost all AP-1A staining co-localized with Rab13. In addition, Rab13 partially co-localized with AP-1B at recycling endosomes in 76 +/- 15% of cells analyzed (Fig. 2-7 B). To confirm this localization, we next analyzed the colocalizations of Rab13, TGN38 (a marker for AP-1A-positive TGN) and TfR (a marker for AP-1B-positive recycling endosomes) in the same cells (Fölsch et al., 2003). Again, Rab13 co-localized with both TGN38 (98 +/- 3% of cells analyzed expressing both markers) and TfR (95 +/- 8% of cells analyzed expressing both markers) with some Rab13 staining not co-localizing with either marker (Fig. 2-7 D). Furthermore, we found no co-localization between Rab13 and the Golgi marker protein, GM130 (Fig. 2-7 C). In addition to TGN/recycling endosomes localization, some Rab13 stained the plasma membrane [arrows in Figs. 2-7 A, C and D (Marzesco et al., 2002)]. Since Rab proteins localize to both
**Figure 2-7: Intracellular localization of Rab13.**

(A-D) MDCK cells grown on coverslips were infected with defective adenoviruses encoding GFP-V5-Rab13 wild type together with defective adenoviruses encoding µ1A-HA (A), µ1B-myc (B), or TGN38 (D). 24 h (D) or 36 h (A-C) post-infection cells were fixed and processed for immunofluorescence staining. µ1A-HA was incubated with mouse anti-HA antibodies; µ1B was labeled with rabbit anti-myc antibodies, and TGN38 with rabbit anti-TGN38 antibodies. Endogenous GM130 (C) was labeled with mouse anti-GM130 antibodies and TfnR (D) was labeled with mouse anti-TfnR antibodies. Subsequently, samples were incubated with the following corresponding secondary antibodies: Alexa 594-labeled goat anti-mouse (µ1A, TfnR), Alexa 594-labeled goat anti-rabbit (µ1B), or Cy5-labeled goat anti-rabbit (TGN38). Specimens were analyzed by confocal microscopy and representative images are shown. Arrows (A, C, and D) indicate plasma membrane localization of Rab13. For quantification, cells co-expressing GFP-V5-Rab13 together with µ1A-HA, µ1B-myc, TGN38 or TfnR were scored for at least partial overlapping staining. Data represent mean values from at least 4 independent experiments with a total of at least 30 cells analyzed (µ1A-HA, 30 cells; µ1B-myc, 65 cells; TfnR, 65 cells; TGN38, 61 cells). Scale bars are 10 µm.
donor and acceptor membranes (Collins, 2003), the partial localization of Rab13 at the TGN and recycling endosomes therefore supports our hypothesis that Rab13 functions at the interface between the TGN and recycling endosomes in addition to its described function at the tight junctions (Marzesco et al., 2002).

Overexpression of Rab13 and not Rab8 or Rab10 disrupts TGN38 localization

Since we found Rab13 localizing at the TGN, we next wondered whether overexpression of Rab13 mutants might have disruptive effects on this compartment. Therefore, we co-injected Rab13Q67L or Rab13T22N cDNAs together with TGN38 cDNA into MDCK cells grown on coverslips. Post-injection, we followed the same protocol (2 h at 39°C and 2 h at 31°C with CHX) we used for co-expression of VSVG in Fig. 2-2 to monitor effects on the TGN. TGN38 is localized at the TGN in cells co-injected with an unrelated plasmid (mRFP-FcR cDNA) and this controls for competing protein synthesis that arises when different plasmids are co-injected (Fig. 2-8 A). However, co-expression of Rab13Q67L or Rab13T22N led to a dispersal of TGN38 localization throughout the cells (Fig. 2-8 B and C). Disruption of TGN38 localization was observed in 97% of cells analyzed for Rab13Q67L and about 80% of the cells analyzed for Rab13T22N (Fig. 2-8 D). In addition, TGN38 might be misrouted into lysosomes since staining intensities also decreased. These experiments were performed as before with the addition of lysosomal and proteasomal inhibitors, ammonium chloride (50 µM) and MG132 (3 mM). As expected the signal intensity of TGN38 was increased, although there was a disruption of the TGN38 localization and TGN38 is dispersed throughout the cytosol (Fig. 2-9). To test whether Rab13 may have a unique function in TGN38 localization, we injected cDNAs
Figure 2-8: Overexpression of Rab13 mutants but not Rab8 or Rab10 mutants disrupts TGN38 localization.

(A-C) MDCK cells grown on coverslips were microinjected with cDNAs encoding TGN38 or mRFP-FcR (A), or TGN38 together with cDNAs encoding V5-Rab13Q67L (B), V5-Rab13T22N (C). Cells were incubated at 39°C and 31°C as described for VSVG in Fig. 2-2. Subsequently cells were processed for immunofluorescence. TGN38 was labeled with mouse anti-TGN38 antibodies and V5-Rab13 mutants were visualized by labeling with anti-V5 antibodies followed by labeling with isotype specific secondary antibodies. Specimens were analyzed by confocal microscopy and representative images are shown. (D) Cells co-injected with TGN38 and Rab cDNAs were scored for correct TGN38 localization (blue bars) or displaced TGN38 localization (red bars). Data represent mean values from at least three independent experiments counting a total of 70 to 80 cells for each condition. Error bars indicate SD. Q, dominant active allele; T, dominant negative allele of the indicated Rab.
Figure 2-9: Disruption of TGN38 localization by Rab13Q67L is not restored by the addition of proteasomal and lysosomal inhibitors.

MDCK cells grown on coverslips were microinjected with cDNAs encoding TGN38 (A), or TGN38 together with cDNAs encoding V5-Rab13Q67L (B). Cells were incubated at 39°C and 31°C as described for VSVG in Fig. 2-2. The 2 h chase at 31°C was performed in the presence of CHX, 0.05 mM MG132, and 3 mM ammonium chloride. Subsequently cells were processed for immunofluorescence. TGN38 was labeled with mouse anti-TGN38 antibodies followed by Alexa 488-labeled goat anti-mouse IgG1 antibodies. V5-Rab13 mutants were visualized by labeling with anti-V5 antibodies followed by Alexa 594-labeled goat anti-mouse IgG2a antibodies. Specimens were analyzed by confocal microscopy and representative images are shown.
encoding Rab8 or Rab10 mutants, together with plasmids encoding TGN38, into MDCK cells
and analyzed TGN38 localization. TGN38 is an AP-1A cargo that is initially delivered from the
TGN to the basolateral membrane but undergoes rapid endocytosis through the recycling
endosomes back to the TGN. As shown in Figs. 2-10 A, C and D and Fig. 2-8 E, neither
Rab8Q67L nor Rab10Q68L or Rab10T23N had any discernible disruption on TGN38
localization. Interestingly, when Rab8T22N was co-injected with TGN38 cDNA, about 50% of
the cells lacked TGN38 staining (Fig. 2-10 B and Fig. 2-8 E). This is especially notable, because
during biosynthetic delivery of AP-1B cargos only Rab8Q67L, but not Rab8T22N showed
disruption of basolateral delivery from recycling endosomes (Ang et al., 2003). However, since
Rab8 localizes to recycling endosomes (Ang et al., 2003), overexpression of Rab8T22N might
interfere with retrieval pathways back to the TGN from early endosomes through recycling
endosomes, this may explain the phenotype we observed with the Rab8 mutant. Regardless, the
effects on TGN38 localization are most pronounced upon overexpression of both Rab13 mutants,
indicating that Rab13’s effects on TGN38 are specific for Rab13.

This disruption effect was specific for TGN38 because overexpression of Rab13 mutants
by microinjection had no discernible effect on the localization of GM130, γ-adaptin (AP-1A and
AP-1B), or TfnR (Fig. 2-11). Interestingly, the TGN marker protein, furin, was also not affected
upon overexpression of Rab13 mutants by microinjection (Fig. 2-11 D and H), indicating that
perhaps only a subdomain of the TGN is affected by Rab13. Prolonged overexpression of Rab13
for 24 h by transient transfection led to a deterioration of the entire Golgi complex, as judged by
a more dispersed staining for GM130, while γ-adaptin or TfnR staining was similar to control
cells (Fig. 2-12). Cells transiently overexpressing Rab13Q67L for prolonged times may
Figure 2-10: Overexpression of Rab8 or Rab10 mutants does not disrupt localization of TGN38.

(A-D) MDCK cells grown on coverslips were microinjected with cDNAs encoding TGN38 together with cDNAs encoding T7-Rab8Q67L (A), T7-Rab8T22N (B), T7-Rab10Q68L (C), or T7-Rab10T23N (D). Cells were incubated at 39°C and 31°C as described for VSVG in Fig. 2-2. Subsequently cells were processed for immunofluorescence. TGN38 was labeled with mouse anti-TGN38 antibodies and Rab8 and Rab10 labeled with anti-T7 antibodies followed by labeling with isotype specific secondary antibodies. Specimens were analyzed by confocal microscopy and representative images are shown. Q, dominant active allele; T, dominant negative allele of the indicated Rab.
Figure 2-11: Overexpression of Rab13 mutants by microinjection has no discernable effect on GM130, AP-1, TfnR or furin localization.

MDCK cells grown on coverslips were microinjected with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N and incubated at 39°C and 31°C as described for VSVG in Fig. 2-2. Cells were then fixed and processed for immunofluorescence staining for V5-Rab13 (anti-V5 tag antibodies [mouse IgG2a]), GM130 (mouse IgG1 antibodies) (A, E), γ-adaptin (mouse IgG1 antibodies) (B, F), TfnR (H68.4, IgG1) (C, G), furin (rabbit polyclonal antibodies) (D, H). Subsequently, cells were incubated with isotype specific, fluorescently labeled secondary antibodies. Specimens were analyzed by confocal microscopy and representative XY images are shown. Scale bars are 10 µm.
Figure 2-12: Overexpression of Rab13 by transient transfection leads to a disruption of the Golgi complex as indicated by GM130.

MDCK cells were grown on coverslips and transiently transfected with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N. 24 h post-transfection, cells were fixed and incubated with antibodies against V5-Rab13 (anti-V5-tag) together with antibodies against GM130 (610822) (A), γ-adaptin (100/3) (B), or TfnR (H68.4) (C). Cells were incubated with fluorescently labeled secondary antibodies: Alexa 488-labeled goat anti-mouse IgG2a (V5) and Alexa 594-labeled goat anti-mouse IgG1 (GM130, γ-adaptin, TfnR). Specimens were analyzed by confocal microscopy and representative XY images are shown. Scale bars are 10 µm.
compensate by expressing more GM130 as judged by the more intense GM130 staining in these cells (Fig. 2-12 A, upper panel). While disruption of the Golgi from prolonged Rab13 overexpression prevented us from using defective adenoviruses for any biochemical analysis, this finding might reconcile our data with the published literature, indicating that disruption of the Golgi complex causes polarity defects.

**Depletion of Rab13 by RNAi in human bronchial epithelial cells leads to disruption of TGN46**

We next confirmed Rab13’s effects on the TGN in a relevant physiological system, by inducing the knock down of Rab13 in the human bronchial epithelial cell line 16HBE14o- (HBE). Like MDCK cells, this cell line expresses Rab13 and µ1B based on RT-PCR, fully-polarized cells sort LDLR-CT27 to the basolateral membrane and grow one single primary cilium (Fig. 2-13), and form ZO-1 containing tight junctions (Kizhatil et al., 2007). However, unlike MDCK cells, these cells are more conducive to RNAi experiments (Kizhatil et al., 2007). We obtained three GFP-tagged shRNA constructs targeting Rab13 and one control construct targeting GAPDH from Open Biosystems. To test knock down capacities, shRNA constructs were transfected into HBE cells together with a dual expression plasmid encoding both monomeric red fluorescent protein (mRFP) and T7-tagged Rab13 from different promoters. 48 h post-transfection, cells were immunolabeled for Rab13 using anti-T7 antibodies and analyzed by confocal microscopy (Fig. 2-14 A). We then determined ratios of Rab13 to mRFP mean pixel intensities of individual cells and expressed the knock down as [%] values from the GAPDH control (see materials and
Figure 2-13: Characterization of the human bronchial epithelial cell line 16HBE140 (HBE).
(A) 16HBE140 cells were grown on fibronectin/collagen coated 10 cm plates and RNA was isolated using Nucleospin RNA II kit (clontech). cDNA was generated using SuperScriptRT III (Invitrogen), which includes reverse transcriptase and oligo(dT) primers. cDNA products were then amplified in a second PCR reaction using gene-specific primers to amplify entire gene products as indicated below. PCR products were run on agarose gels. Ethidium bromide stained DNA bands were visualized using the chemi genius bioimaging system from Syngene. (B) 4 x 10^5 16HBE140 cells were seeded onto fibronectin/collagen coated 12-mm transwell filters and incubated at 37°C. 3 days post-seeding, cells were infected with defective adenoviruses encoding LDLR-CT27. 2 days post-infection, LDLR-CT27 at the surface was stained with C7 antibodies. (C) 4 x 10^5 16HBE140 cells were seeded onto fibronectin/collagen coated 12-mm transwell filters and incubated for 10 d. Primary cilia were stained with antibodies against acetylated tubulin followed by Alexa 488-labeled goat anti-mouse antibodies. The arrow marks the position of the filter. (B, C) Specimens were analyzed by confocal microscopy and representative XY and XZ sections are shown.
Figure 2-14: Knock down of Rab13 in HBE cells leads to a disruption of TGN46 and GM130 localization.

(A) HBE cells grown on collagen and fibronectin coated coverslips were transfected with plasmids encoding various shRNA constructs (upper panel GAPDH, middle panel Rab13 #1, and bottom panel Rab13 #3) together with dual expression plasmids encoding mRFP and T7-Rab13WT. 48 h post-transfection, cells were fixed and stained with anti-T7 antibodies to visualize Rab13 (in blue, using goat anti-mouse Cy5-labeled secondary antibodies). All specimens were analyzed using the same confocal settings and representative images are shown. The ratio of T7-Rab13 to mRFP staining was determined and [%] values of knock down were calculated as described in materials and methods from three independent experiments counting at least a total amount of 30 individual cells (errors indicate SD). (B) HBE cells were seeded onto coated coverslips and transfected with plasmids encoding shRNA constructs (upper panel GAPDH, middle panel Rab13 #1, and bottom panel Rab13 #3). 48 h post-transfection, cells were fixed and labeled with anti-TGN46 antibodies (in red, using goat anti-rabbit Alexa 594-labeled secondary antibodies) and anti-GM130 antibodies (in blue, using goat anti-mouse Cy5-labeled secondary antibodies). Specimens were analyzed by confocal microscopy and representative images are shown. At least 50 cells from at least three independent experiments were scored for TGN46 or GM130 staining. Values indicate the percent of cells with a disruptive phenotype expressing the shRNA construct. Errors are SD. Cells transfected with shRNAs are outlined in white. Scale bars are 10 µm. Abbreviations: KD, knock down; DP, disruption phenotype.
methods for details). This was necessary, because we do not have antibodies against endogenous Rab13. We found that constructs Rab13 #1 and Rab13 #3 knocked expression down to 13% and 18% of control levels (Fig. 2-14 A), respectively, whereas construct #2 was not effective (data not shown). Next, we transfected shRNA constructs Rab13 #1 and Rab13 #3 as well as GAPDH constructs into HBE cells and stained transfected cells with antibodies against endogenous TGN46 and GM130 48 h post-transfection. We found that a knock down of Rab13 resulted in a disruption and/or loss of TGN46 staining in about 57% of cells analyzed for construct Rab13 #1 and in about 80% of cells analyzed for construct Rab13 #3 (Fig. 2-14 B). These data indicate that Rab13 is indeed needed for the maintenance of TGN38/TGN46 localization at the TGN. Interestingly, as was the case with prolonged Rab13 overexpression of both mutants, knock down of Rab13 also led to a disruption/loss of GM130 staining in 43% of cells analyzed for construct Rab13 #1 and 62% of cells analyzed for construct Rab13 #3 (Fig. 2-14 B). Recently it was shown that Rab13 knock down in MDCK cells also led to a disruption of tight junction integrity (Yamamura et al., 2008). Therefore it seems that prolonged overexpression and knock down of Rab13 share the same phenotypes in disrupting TGN38/TGN46 and GM130 staining at the TGN/Golgi together with impairing tight junctions. This is in contrast to acute overexpression by microinjection, which leaves the tight junction (and the Golgi) mainly intact.

Discussion

Collectively, we found that movement of cargo proteins that travel to the plasma membrane via recycling endosomes (LDLR-CT27, VSVG, and A-VSVG) was stalled following overexpression of Rab13 mutants. Cargos not traversing the recycling endosomes, such as the
basolateral cargos LDLR(Y18A) and FcR and the apical cargo HA were not changed. Since both VSVG and its apical variant A-VSVG were blocked, Rab13 appears to function between the TGN and recycling endosomes, as opposed to regulating post-recycling endosome trafficking pathways, which are different for VSVG and A-VSVG. As the disruption of surface delivery for the cargo proteins is specific to Rab13, we have uncovered a unique role for Rab13 in mediating protein surface delivery in a polarized manner.

The partial localization of Rab13 at the TGN and recycling endosomes supports the hypothesis that Rab13 functions at the interface of the two organelles, in addition to its described function at the tight junctions (Marzesco et al., 2002). Previously published studies of Rab13 reflected defects in the tight junctions, exemplified by disrupted ZO-1 and gp58 staining. However, these data were based on prolonged overexpression of Rab13 mutants via defective adenoviruses for more than 24 h, or on data seen in stable cell lines expressing GFP-Rab13 mutants (Marzesco et al., 2002; Morimoto et al., 2005). We found that prolonged overexpression of Rab13 mutant alleles led to disruption of the Golgi complex as seen in Figure 2-12. It is possible that over time disruption of the Golgi complex may then lead to polarity defects. In addition, overexpression of Rab13Q67L might lead to tight junction defects due to enhanced delivery of activated Rab13 to the tight junctions via the biosynthetic pathway.

Importantly, our study is the first to demonstrate any phenotypes for the Rab13T22N allele. In this study, we report similar results for the dominant active, Rab13Q7L mutant and the dominant negative, Rab13T22N mutant. Both exhibited a lack of surface delivery for the cargo proteins transported from the TGN to the recycling endosomes (LDLR-CT27, VSVG, and A-VSVG) in MDCK cells (Figs. 2-2, 2-5, 2-6). In fact, it is possible that the dominant active mutant of Rab13, Rab13Q67L performs more like a dominant negative construct, inactive and
incapable of transport. Previously, Schuck et al. suggested that the phenotypes resulting from the “active” Rab10 mutants in fact behaved as dominant “negative” (i.e. inhibiting the endogenous Rab10) (Schuck et al., 2007). This would explain the enlarged Golgi observed after staining with GM130 in Figure 2-12. The TGN and the preceding Golgi cisternae are flexible organelles whose membranes have been shown to expand and hold exiting cargo when exit is inhibited. (Bard and Malhotra, 2006; Toomre et al., 1999). We report phenotypes with both mutant alleles unlike any other research group and we conclude that Rab13’s primary function is to control membrane trafficking between the TGN and recycling endosomes.

Among the closest mammalian homologues of Rab13 are Rab8 and Rab10. Previously, overexpression of the constitutively active mutant Rab8Q67L (but not Rab8T22N) by microinjection of cDNAs into polarized MDCK cells resulted in the apical missorting of VSVG (Ang et al., 2003). In MDCK cells grown on coverslips, infection with defective adenoviruses expressing Rab10Q68L resulted in VSVG trapped in the Golgi as marked by colocalization with the resident Golgi protein, giatin. Again, the results for VSVG were only seen for the constitutively active mutant and not for the dominant negative mutant, Rab10T23N (Schuck et al., 2007). In this study, we report the inhibition of surface delivery of VSVG for both Rab13 mutants. In comparison with previous studies the demonstration of a disruptive phenotype for both is unique and strengthens our findings for the involvement of Rab13 in trafficking.

Rab13 activity clearly differs from that of its homologues Rab8 and Rab10 in two key aspects. First, Rab13 is critical to protein sorting through the TGN-recycling endosomes pathway. Overexpression of Rab13 mutants inhibited surface delivery in the biosynthetic pathway of both apical and basolateral cargo that moves through recycling endosomes as opposed to apical missorting (Ang et al., 2003; Schuck et al., 2007). Therefore Rab13 appears to
operate at an earlier membrane trafficking step distinct from Rab8 or Rab10. Second, in contrast to Rab8Q67L and Rab10Q68L, Rab13Q67L is important for TGN38 localization and TGN integrity. Neither Rab8Q67L, Rab10Q68L, nor Rab10T23N had any discernible disruption phenotype of TGN38 localization. It is interesting to note that Rab13 had no effect on the localization of furin as seen in Figure 2-11 D and H. Furin is an AP-1A cargo transmembrane protein that recycles between the TGN and the early endosomes. In comparison, TGN38 is an AP-1A cargo transmembrane protein that is transported to the basolateral membrane after passing through the recycling endosomes. TGN38 is quickly endocytosed from the basolateral membrane and is predominantly localized to the TGN (Mallet and Maxfield, 1999). So in fact, again Rab13 mutant alleles only have a disruptive effect on the cargo proteins, like TGN38 transported through the recycling endosomes. It appears that Rab13 is essential for organizing a subdomain of the TGN necessary for surface delivery of transmembrane proteins transported through the recycling endosomes to the plasma membrane and therefore is essential to overall organization of individual epithelial cells and epithelial monolayers.

In the future it will be interesting to investigate the molecular mechanisms behind Rab13’s function in cargo transport. Currently very little is known about effector proteins of Rab13. For instance, the proteins functioning as guanine nucleotide exchange factors (GEFs) or GAPs in the GTPase cycle of Rab13 are unknown. One effector protein of Rab13 that has been identified is PDEδ. PDEδ was isolated as the δ-subunit of the rod-specific phosphodiesterase, but was later shown to be a structural homologue of RhoGDI and is capable of extracting prenylated Rab13 from membranes (Marzesco et al., 1998). Previously it has been shown that PDEδ interacts with the Arf-like protein, Arl3, and Arl3 recruits Arl1, which is necessary for TGN maintenance (Linari et al., 1999a; Lu et al., 2001; Panic et al., 2003). It will be interesting to
further understand Rab13’s role at the TGN and the possible interaction of Rab13 and other TGN maintenance proteins.

**Materials and Methods**

**Cloning, RNAi constructs and adenoviruses**

Rab13 clone by Ruth Collins was used as a template to generate V5-tagged Rab13 using the following N- and C-terminal primers: 5’-

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GCAGATCTATGGGAAAGCCAATACCAAAACCCCCTTTATTAGGATTAGACAGCACAGCC
AAAGCCTACGACCACCTCTCTTC-3’ and 5’-
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GCGCAAGCTTTTCAGCCAGGGAGCACTTGTTGCT-3’, respectively. The PCR products were cloned as BglII/HindIII fragments into the microinjection vector pRKV or the adenovirus shuttle vector pShuttle-CMV. EGFP-V5-tagged Rab13 was cloned by inserting DNA encoding EGFP into the BglII site of pShuttle-CMV as BglII/BamHI fragments. T22N and Q67L mutations were introduced into Rab13 by Quikchange site-directed mutagenesis (Stratagene) using V5-Rab13 in pRKV as a template and matching sense/antisense primer pairs. The sense primer for T22N was 5’-GGGTGGGGCAAGAATTGTCTGATCAT-3’, and the sense primer for Q67L mutagenesis was 5’-ACACGGCTGGCCTAGAGCGTTCAA-3’. T7-tagged Rab13 was generated using the following N-terminal primer 5’-

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GCGCAGATCTATGGCTAGCATGACTGGTGCGCTACGAAATGGGGTCCTAGACAGTTCAA-3’ and the same C-terminal primers and template as above. T7-Rab13 PCR products were cloned as BglII/BamHI fragments behind the CMV promoter in pBUDCE4. Subsequently, mRFP was cloned behind the EF-1α promoter of pBUDCE4 as KpnI/BamHI PCR fragments. mRFP was amplified using mRFP-FcR as template and the following N- and C-
terminal primers: 5’-GCGCGGTACCATTGCCCTCTCCGAGGACGTATC-3’ and 5’-GCGCGGATCTCTAGGGCCGGTGGAGTGGGCAGCCCTC-3’, respectively.

Plasmids expressing canine Rab10 and mutant Rab10s were obtained from Dr. Kai Simons (Dresden, Germany). We subsequently used these plasmids as templates to amplify T7-tagged Rab10 using the following N- and C-terminal primers: 5’-GCAGATCTATGGCTAGCATGACTGGTGGACAGCAAATGGGTGCGAAGAAGACGTACGACCTGCTTTTC-3’ and 5’-GCGCAAGCTTTCAGCAACATTTGCTCTTCCAGCC-3’, respectively. PCR products were cloned as BglII/HindIII fragments into pRKV.

T7-tagged Rab8 mutants in pRKV were as described previously (Ang et al., 2003). HA in vector pCAGGS was obtained from Dr. Robert Lamb (Northwestern University). CFP-VSVG in pEYFP-N1 was obtained from Dr. Derek Toomre (Yale University), GFP-A-VSVG in pRKV, as well as TGN38, mRFP-FcR, LDLR-CT27 and LDLR(Y18A) in pShuttle-CMV were as previously described (Ang et al., 2003; Fields et al., 2007). Defective adenoviruses encoding \( \mu1A-HA \) or \( \mu1B-myC \) were as described previously (Fields et al., 2007). Defective adenoviruses encoding EGFP-V5-Rab13 were generated as described previously in He et al., "A simplified system for generating recombinant adenoviruses" (He et al., 1998).

RNAi constructs targeting human Rab13 (oligo IDs V2LHS_171155, V2LHS_278139, and V2LHS_171156 with the respective catalogue numbers RHS4430-98526311, RHS4430-99148915, and RHS4430-98896339) or human GAPDH (catalogue number RHS4371) were purchased from Open Biosystems. We obtained shRNAamir constructs in the lentiviral vector pGIPz, which expresses turboGFP-tagged shRNAs. The sequences were the following for construct 1:

\[
\text{TGCTGTTGACAGTGAGCGAGGATGAGAAATCTTTCGAGAATAGTGAAGCCACAGAT} \]

...
GTATTCTCGAAAGATTTCTCATCCGTGCCTACTGCCTCGGA (V2LHS_171155),

construct 2:
TGCTGGACTGAGCGAGGCATGCTGAGGGTAAATAGTGAAGCCACAGAT
GTATTACCCTACCTATGTGACCCTACTGCCTCGGA (V2LHS_278139), and

construct 3:
TGCTGGACAGTGAGCGACCTCCCAGTACTGACCTGAAATAGTGAAGCCACAGAT
GTATTTTCAGGTCAGTACTGGGAGGCTGCCTCGGA (V2LHS_171156).

Cell culture

MDCK cells were cultured in MEM with the addition of 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin, and 7% (vol/vol) fetal bovine serum at 37°C and 5% CO2. 16HBE14o-cells were purchased from Dr. Dieter Gruenert (California Pacific Medical Center) and cultured on fibronectin/collagen coated plates or coverslips in MEM with the addition of 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin, and 10% (vol/vol) fetal bovine serum at 37°C and 5% CO2. The coating solution contained LHC basal medium with 10 mg/100 ml bovine serum albumin, 3 mg/100ml bovine collagen I, and 1 mg/100ml fibronectin.

For microinjection experiments of polarized cells, 4 X 10^5 cells were seeded per 12-mm clear filter (0.4 µm pore size; Corning-Costar Transwell units) and cultured for 3-4 d with changes of the medium in the basolateral chamber every day. After excision of the filter from the filter holders, cells were placed in HEPES-buffered (50 mM) media and 0.2 mg/ml cDNAs were microinjected into the nuclei of the cells using an Eppendorf Femtoject (Injectman® NI2)
mounted on an inverted microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) with a heated stage.

For injection of ts045 mutants of VSVG and A-VSVG, cells were injected at 39°C, after injection, cells were incubated for 2 h at 39°C followed by 2 h at 31°C in the presence of 50 mM HEPES-buffered media and 0.1 mg/ml CHX with or without the addition of 0.05 mM MG132 and 3 mM ammonium chloride. Other cargos were injected at 37°C, followed by a 1 h incubation at 37°C, 2-4 h at 20°C in 50 mM HEPES-buffered media, which was followed by a chase at 37°C for 1-2 h in the presence of 0.1 mg/ml CHX. Subsequently, cells were processed for immunofluorescence microscopy (see below).

For immunofluorescence experiments with cells grown on coverslips, cells were seeded on Alcian blue or fibronectin/collagen-coated coverslips and cultured for 2-4 d. Infection of cells with defective adenoviruses was performed as described previously (Fields et al., 2007). Transient transfections were done using LipofectAMINETM 2000 (Invitrogen) according to the manufacturer’s protocol.

**Antibodies**

Mouse monoclonal antibodies recognizing GM130 (610822) or TGN38 (T69020) were obtained from Transduction Laboratories. Mouse monoclonal antibodies recognizing acetylated tubulin (6-11B-1) or γ-adaptin (100/3) were from Sigma. From Zymed we purchased mouse monoclonal antibodies recognizing ZO-1 (33-9100). Monoclonal antibodies against the T7-epitope (69522-3) were from Novagen, antibodies against the HA-epitope (16B12) from Babco/Covance, and antibodies recognizing the V5-epitope (336) were generated by Dr. Richard
Rabbit polyclonal antibodies against furin (PA1-062) were purchased from Affinity Bio Reagents, anti-GFP antibodies (ab90) and anti-TGN46 antibodies (50595) were from Abcam, and anti-myc antibodies (A-14) were from Santa Cruz Biotech. Polyclonal antibodies against GM130 or TGN38 were a gift from Dr. Graham Warren (Vienna, Austria). Goat polyclonal antibodies recognizing the ectodomain of HA (anti-H3 [A/HongKong/1/68], # V-314-591-157) can be obtained from NIAID.

Hybridomas producing antibodies against δ-adaptin (SA4) were generated by Dr. Andrew Peden (University of Cambridge, UK) and obtained from the Developmental Studies Hybridoma Bank (DSHB). Hybridomas producing antibodies recognizing gp58, FcR (2.4G2), LDLR (C7), human TfnR (H68.4), or VSVG (TK1) were as described previously (Fields et al., 2007). Secondary antibodies labeled with Alexa dyes were purchased from Molecular Probes. Cy5-labeled goat anti-rabbit or goat anti-mouse antibodies were from Jackson ImmunoResearch, and Cy5-labeled goat anti-mouse IgG2a antibodies were purchased from Southern Biotech.

**Immunofluorescence microscopy**

In general, cells were fixed in 3% PFA for 15 min at RT followed by an incubation in PBS$^{2+}$ for 5 min. Subsequently, cells were incubated for 1 h in a blocking/permeabilization solution (BPS) (2% [wt/vol] BSA, 0.4% [wt/vol] saponin in PBS$^{2+}$) with 10% [vol/vol] goat serum. Cells were then incubated for 1 h with primary antibodies in BPS, followed by 5 washes over 30 min with BPS. Finally, cells were incubated for 1 h with secondary antibodies in BPS,
followed by 5 washes in BPS. Cells were mounted in a solution containing 10% [wt/vol] DABCO and 50% [wt/vol] glycerol. For cell surface staining, cells were incubated with primary antibodies for 1 h on ice followed by washes with ice-cold PBS$^{2+}$ prior to fixation.

Specimens were analyzed using a confocal microscope (Microsystem LSM 510; Carl Zeiss MicroImaging, Inc.) equipped with a 63 X water immersion lens. Images were combined and enhanced using Adobe Photoshop®.

For quantification of shRNA knock down, HBE cells were co-transfected with shRNA constructs and pBUDCE4 encoding mRFP and T7-Rab13 24 h post-seeding. 48 h post-transfection, cells were fixed, permeabilized and stained for T7-Rab13. Images of specimens were taken under the same confocal settings and mean pixel intensities were obtained using Zeiss510 ConfoCor 3 software. We then determined the ratio between T7-Rab13 and mRFP signals. The mean ratio of cells co-expressing a control shRNA construct targeting GAPDH was set 100% and the values of knock down for the Rab13 shRNA constructs was determined as [%] values of the control.
CHAPTER 3: LOCALIZATION OF NGCAM IN POLARIZED EPITHELIAL CELLS
INTRODUCTION

Polarity in differentiated cells is established with elaborate, distinct plasma membrane domains that differ in their protein and lipid components. Highly polarized cells, such as epithelial cells, differentiate and maintain apical and basolateral membrane domains while neurons establish dendrites and axons. The distinct membrane compositions are maintained by diffusion barriers found at the tight junctions in epithelial cells (Shin et al., 2006) and the axonal initial segment in neurons (Winckler et al., 1999) (Fig. 3-1). Like epithelial cells, neurons secrete transmembrane proteins in a polarized manner from the trans-Golgi network (TGN) during biosynthetic delivery, and the recycling endosomes serve as a sorting center after internalization. Similar to epithelial cells, sorting signals are encoded in the membrane proteins themselves.

Although some studies of membrane trafficking in neurons have been completed, less information is known about neuronal molecular machinery than the machinery utilized by epithelial cells. The current knowledge about polarized sorting is derived from studies involving the kidney epithelial cell line, MDCK. In 1990, Dotti and Simons proposed that the targeting of proteins to the apical and the axonal domains might use related signals and machinery, and that basolateral and somatodendritic targeting also share common features (Dotti and Simons, 1990). For example, both basolateral and somatodendritic sorting signals frequently rely on tyrosine-based sorting signals encoded in the cytoplasmic tails of transmembrane proteins (Fölsch, 2005; Silverman et al., 2005; West et al., 1997). The recycling transmembrane protein transferrin receptor (TfnR) is endogenously expressed in epithelial cells and neurons. In epithelial MDCK cells, TfnR is biosynthetically transported from the TGN to the plasma membrane and then
Figure 3-1: Comparison of polarized domains in neurons and epithelial cells.

The neuron and the epithelial cell have comparable membrane domains. The somatodendritic and axonal membranes are separated by the initial segment that acts as a diffusion barrier. In epithelial cells the basolateral and apical membrane domains are physically separated by the tight junction proteins. Some of the same sorting machinery to the different plasma membrane domains is conserved between cell types.
transported from the recycling endosomes to the plasma membrane and often are endocytosed via clathrin-coated pits. The internalization is dependent on the adaptor protein (AP) complex AP-2 as well as a tyrosine motif involving amino acid 20 in the cytoplasmic tail (Robinson and Bonifacino, 2001). In neurons, TfnR is localized to dendrites and is strictly excluded from the axon. This same tyrosine motif was found to be critical in the endocytosis and dendritic targeting of TfnR, implying there are some overlapping signals and similar molecular mechanisms (Anderson et al., 2005). Typically tyrosine-based signals are recognized by cytosolic AP-1 through AP-4 (Fölsch, 2005) (Nakatsu and Ohno, 2003; Rodriguez-Boulan et al., 2005). In some cases, tyrosine-based signals bind more than one adaptor complex and are active as both basolateral and endocytosis signals (Fölsch, 2005). Epithelial cells such as MDCK cells express a subclass of the AP-1 complex, AP-1B, that is not found in neurons or hepatocytes (Ohno et al., 1999). AP-1B is important for the correct sorting of basolateral cargo proteins from recycling endosomes to plasma membrane (Fölsch et al., 2003; Gan et al., 2002; Ohno et al., 1999). However, AP-4 is thought to sort proteins directly from the TGN to the basolateral membrane during biosynthetic delivery and there may be other adaptors that are able to compensate for the lack of AP-1B in neurons. Thus far, neuronal transport of transmembrane proteins to the somatodendritic domain is found to be mediated by cytoplasmic tail signals and these signals are frequently closely related to basolateral targeting determinants (Cheng et al., 2002; West et al., 1997). Indeed, some signals and machinery involved in polarized trafficking are conserved between cell types, but there are cell-specific mechanisms as well. Evidence for cell-specific sorting makes mechanistic conservation an equally interesting area of study.

Apical and axonal signals have been mapped to the extracellular, transmembrane, and cytoplasmic domains of proteins. Because these targeting signals are diverse, the machinery
recognizing apical and axonal targeting signals is largely unknown. As in epithelial cells, axonal proteins have membrane-anchoring domains that partition into glycolipid raft microdomains. Two examples of axonal cargos that require lipid rafts for sorting are the GPI-linked proteins, Thy1 and PrP<sup>C</sup> (Galvan <i>et al.</i>, 2005; Ledesma <i>et al.</i>, 1998). Several axonal signals have been mapped to the cytoplasmic domains of these cargo proteins, but no consensus sequence has emerged.

Unlike MDCK cells, neurons are currently thought to have two distinct pathways of cargo delivery. Along the first path, axonal delivery is likely regulated by competence for fusion. This mechanism reflects a pathway in which there is not necessarily discrimination for sorting at the TGN, but instead discrimination is at the target site. An anchoring or retention signal would hold the cargo protein in the membrane domain and subsequently the cargo cannot be endocytosed anymore. Alternatively, a targeting signal or sorting signal may sort proteins into distinct vesicles at the TGN, which are destined for the axonal or somatodendritic domains (Winckler, 2004) (Fig. 3-2). Recent studies, including data found within this thesis, support the idea that signal-mediated sequences have a crucial role in neuronal transport.

The evolutionary conservation of sorting mechanisms across different cell types is an intriguing prospect, so it is of particular interest to characterize the transport of membrane proteins expressed endogenously by both epithelial cells and neurons. In addition to TfnR, the cell adhesion molecule NgCAM is expressed in epithelial cells and neurons. NgCAM is a member of the immunoglobulin superfamily and it is the chick homologue of human L<sub>1</sub> cell adhesion molecule (Walsh and Doherty, 1997). The cytoplasmic domain of NgCAM contains an Y<sub>33</sub>RSLLE tyrosine motif, a glycine-rich region, an ankyrin-binding domain, and a serine-rich region. In neurons, NgCAM reaches the axonal plasma membrane after insertion into the
Figure 3-2: Two distinctive pathways of cargo delivery.
Cargo transported out of the TGN is thought to travel in two possible paths. (1) Axonal and somatodendritic cargo are incorporated into distinct vesicles at the TGN. (2) Axonal cargo is sorted until it is recognized by an anchoring or retention signal. And thus, the cargo cannot be endocytosed.
somatodendritic domain (Wisco et al., 2003). The Y₃₃RSLE motif of NgCAM is necessary for initial basolateral and somatodendritic targeting (Anderson et al., 2005; Wisco et al., 2003). Bettina Winckler’s laboratory has found that amino acids 44-59 of the cytoplasmic tail comprise an axonal targeting signal, and that amino acids 66-114 form an axonal retention signal in hippocampal neurons (Yap et al., 2008). In addition, amino acids 66-114 have been identified as an ankyrin binding domain and the domain contains a specific ankyrin-binding motif, FIGQY₈₆. A tyrosine to aspartic acid mutation at amino acid 86 has been shown to disrupt ankyrin B binding. Ankyrin functions as a protein linker between integral membrane proteins and the spectrin-based cytoskeleton. The comparison of molecular machinery between neurons and epithelial cells is made more complex when we consider that ankyrin B is located in the axons of neurons but is found in the basolateral membrane of MDCK cells (Rubtsov and Lopina, 2000).

In MDCK cells, NgCAM is also transcytosed. Initial basolateral transport is mediated by the Y₃₃RSLE motif, which is decoded by the AP-1B complex. It appears that after arrival at the basolateral surface, the interaction site is silenced by phosphorylation of Tyr₃₃ and AP-1B is unable to recycle NgCAM back to the basolateral membrane after internalization. This phosphorylation also slows down endocytosis. These events result in the transcytosis of NgCAM due to a cryptic apical targeting signal in its extracellular domain (Anderson et al., 2005; Sampo et al., 2003; Wisco et al., 2003). In theory, this means that the cytoplasmic tail is dispensable for axonal/apical accumulation. However, signals in the cytoplasmic tail of NgCAM are required for trafficking via the transcytotic route in hippocampal neurons. This includes the signal encompassing tyrosine 33 (Wisco et al., 2003).
Trafficking along the transcytotic pathway requires the execution of multiple targeting signals that must be read and executed sequentially, presumably in different locations. Therefore, in the initial step of transcytosis a somatodendritic/basolateral signal is active and cis-dominant over an axonal/apical signal. After somatodendritic/basolateral delivery and endocytosis, the axonal/apical signal becomes active in endosomes whereas the somatodendritic/basolateral signal is turned off. The transcytotic model predicts that the ‘recessive’ axonal signals may be executed in the biosynthetic pathway if the somatodendritic/basolateral signal is deleted or mutated. In this work, we identify a second axonal targeting signal located in the cytoplasmic tail that promotes apical trafficking in MDCK cells. This work compliments similar studies performed in hippocampal neurons (Yap et al., 2008). Transcytotic routing in neurons and MDCK cells is therefore dependent on the presence of hierarchical regulation of multiple sorting signals in the cytoplasmic tail of NgCAM.

RESULTS

Generation of a NgCAM chimeric protein

The focus of our study was the targeted mutation of the cytoplasmic tail of the NgCAM protein that is 114 amino acids long and includes an endocytosis motif, a glycine-rich region, an ankyrin binding motif, and a proline-rich region (Anderson et al., 2005) (Wisco et al., 2003). Because the ectodomain of NgCAM contains a sufficient axonal sorting signal, it is almost impossible to identify cytoplasmic signals when the extracellular domain is present. Therefore, we generated chimeric proteins containing the extracellular and transmembrane domains of low density lipid receptor (LDLR) conjugated to different segments of the NgCAM cytoplasmic tail,
see Figure 3-3. To identify targeting signals, a series of deletions on the C-terminal cytoplasmic region of NgCAM were fused in frame downstream of the extracellular and transmembrane domains of LDLR for studies in neurons. However, LDLR minus its complete cytoplasmic tail is apically enriched in MDCK cells. Therefore, we made chimeras using a LDLR carboxy-terminal truncation (CT27) for studies in MDCK cells. This construct localizes to the basolateral membrane due to the motif surrounding tyrosine 18 of LDLR and is AP-1B-dependent for basolateral sorting (Fields et al., 2007; Matter et al., 1992). The basolateral targeting signal of the LDLR ectodomain can, however, be useful to help determine the roles of different regions of the NgCAM cytoplasmic tail. We hypothesized that portions of the NgCAM tail encoded apical sorting signals. Addition of these signals onto the truncated LDLR protein would reverse polarity and cause proteins that normally localize to the basolateral membrane to traffic to the apical domain.

An apical targeting signal in the NgCAM cytoplasmic tail residues 45-59 is sufficient to promote apical targeting

When NgCAM is expressed in MDCK cells, it localizes to the apical surface (Anderson et al., 2005) and reaches the plasma membrane primarily by transcytosis (Anderson et al., 2005; Hua et al., 2006). In the current study, NgCAM chimeric constructs were transiently transfected into fully polarized MDCK cells and expressed for 30 h. The transfected constructs were visualized by surface staining with either anti-NgCAM (8D9) or anti-LDLR antibodies (C7). After surface staining, the cells were fixed, permeabilized and stained for the endogenous basolateral marker protein, gp58. We find complete apical enrichment of NgCAM
Figure 3-3: Schematic depiction of NgCAM and the LDLR/NgCAM chimera.

(A) A schematic representation of NgCAM protein, including the ectodomain, transmembrane domain and the cytoplasmic tail, which includes multiple targeting signals. (B) A schematic representation of the LDLR C-terminal truncation used as the anchor for different portions of the NgCAM cytoplasmic tail.

* Ankyrin B is axonal in neurons but basolateral in MDCK cells
in MDCK cells (Fig. 3-4 A).

The basolateral localization of the LDLR-CT27 construct makes it conducive to monitor the resulting apical sorting effects, if any is found, in domains of the NgCAM cytoplasmic tail. We tested the LDLR-CT27 construct without any portions of the NgCAM tail. As described previously (Matter et al., 1992), LDLR-CT27 localized to the basolateral membrane and there was co-localization with the basolateral protein gp58 (Fig. 3-4 B). We first tested the distal half of the NgCAM tail (44-114) in order to assess additional targeting signals. LDLR-CT27+NgCAM 44-114 showed non-polarized distribution with apical and basolateral membrane enrichment (Fig. 3-4 C). Suggesting an apical sorting signal is located in the NgCAM cytoplasmic tail amino acids 44-114.

Successive truncations from the carboxy terminus were generated to determine the minimal sequence requirements for apical enrichment (shown in Figure 3-3). We found that LDLR-CT27+NgCAM 44-66 and LDLR-CT27+NgCAM 44-59 partially localize to the apical membrane in polarized MDCK cells (Figs. 3-4 D-E). Other regions of the NgCAM tail did not cause apical sorting indicating that longer portions of the tail are not necessary. The observation that residues 44-59 of the NgCAM tail direct the LDLR/NgCAM chimeric protein to the apical surface suggest that we identified the shortest signal sequence critical for apical delivery.

**Further analysis of NgCAM cytoplasmic tail residues 66-114**

If the cytoplasmic tail of NgCAM 44-59 is capable of directing localization to the apical membrane, we would hypothesize that removing those residues from the protein would result in basolateral localization of the protein. In fact upon transient transfection, LDLR-
Figure 3-4: An apical targeting signal in the NgCAM cytoplasmic tail residues 45-59 is sufficient to promote apical targeting.

(A-E) Polarized MDCK cells were transiently transfected with cDNAs encoding NgCAM (A), LDLR-CT27 (B), LDLR-CT27+NgCAM 44-114 (C), LDLR-CT27+NgCAM 44-66 (D) and LDLR-CT27+NgCAM 44-59 (E) and incubated at 37°C for 30 hours. (A) Cells expressing NgCAM were surface stained with anti-NgCAM antibody (8D9). Cells were fixed, permeabilized and incubated with secondary antibodies labeled with Alexa 594. (B-E) The transfected constructs were visualized by surface staining with anti-LDLR antibodies (C7). After surface staining, cells were fixed, permeabilized and stained for an endogenous basolateral marker protein using the anti-gp58 antibody. Cells were then incubated with secondary antibodies labeled with Alexa 488 (gp58) and Alexa 594 (LDLR-CT27 constructs). Specimens were analyzed by confocal microscopy, and representative XZ images are shown. (F) Schematic depiction of LDLR-based NgCAM cytoplasmic tail chimeras.
CT27+NgCAM 66-114 localization is observed at the basolateral membrane colocalizing with gp58 with no apical enrichment (Fig. 3-5 A). These results confirm that NgCAM 44-59 is necessary to cause the chimera to sort apically. This result may be due in part to the ankyrin binding motif FIGQY located in the cytoplasmic tail of NgCAM. However, direct comparisons of protein sorting signals between hippocampal neurons and MDCK cells are complicated due to the fact that Ankyrin B is localized to the basolateral membrane in MDCK cells and the axonal membrane in neurons. With the removal of the ankyrin binding domain in the chimera, LDLR-CT27+NgCAM 66-114(ΔFIGQY), we in fact do see apical enrichment at the apical membrane (Fig. 3-5 B). It appears that removal of putative MDCK basolateral membrane binding signals results in apical enrichment, indicating that the region of amino acids 66-114 contains a second, less potent apical sorting signal.

The full length LDLR cytoplasmic tail contains two basolateral sorting determinants (Matter et al., 1992). The proximal signal located closer to the transmembrane domain is a FXNPXY motif, with a tryrosine residue at position 18 similar to the YRSL motif of NgCAM, and is sorted through the recycling endosomes (Bonifacino and Traub, 2003). The distal targeting determinant is considered a non-canonical tyrosine-based motif, GYSY and is AP-1B-independent. LDLR-CT27 contains only the proximal basolateral sorting signal Y₁₈ and is localized in the axons of neurons but is found at the basolateral membrane of MDCK cells. We next introduced the Y₁₈A mutation into the LDLR-CT27+NgCAM 66-114 chimeric. As expected, expression of LDLR-CT27(Y₁₈A)+NgCAM 66-114 in polarized MDCK cells caused an enrichment of the mutant protein at the apical membrane with very little expression on the
Figure 3-5: The distal portion of NgCAM cytoplasmic tail, NgCAM 66-114 localizes to the basolateral membrane with no apical enrichment.

(A-D) Polarized MDCK cells were transiently transfected with cDNAs encoding LDLR-CT27+NgCAM 66-114 (A), LDLR-CT27+NgCAM 66-114(ΔFIGQY) (B), LDLR-CT27(Y18A)+NgCAM 66-114 (C), or LDLR-CT27(Y18A)+NgCAM 66-114(ΔFIGQY) (D) and incubated at 37°C for 30 hours. The transfected constructs were visualized by surface staining with anti-LDLR antibodies (C7). After surface staining, cells were fixed, permeabilized and stained for an endogenous basolateral marker protein using the anti-gp58 antibody. Cells were then incubated with secondary antibodies labeled with Alexa 488 (gp58) and Alexa 594. Specimens were analyzed by confocal microscopy, and representative XZ images are shown. (E) Schematic depiction of LDLR-based NgCAM cytoplasmic tail chimeras.
basolateral membrane (Fig. 3-5 C). Since some expression was still observed at the basolateral membrane, we designed another truncation mutant with all basolateral membrane signals removed and tested whether the NgCAM tail would result in total apical sorting. In fact, we found the LDLR-CT27(Y18A) + NgCAM 66-114(ΔFIGQY) chimera is localized exclusively at the apical membrane (Fig. 3-5 D). With the removal of MDCK basolateral signaling determinants, we find that indirectly there is indeed a putative apical targeting signal in the NgCAM cytoplasmic tail 66-114.

**DISCUSSION**

Despite the complexities of comparing the protein sorting mechanisms utilized by different cell types, such as epithelial cells and neurons, similarities have been found that suggest that some of the mechanisms are conserved while others are cell specific. Previous studies have found that NgCAM is directed from the TGN to the basolateral surface as an AP-1B-dependent cargo protein in MDCK cells. After this initial transport, the YRSL basolateral targeting motif is then inactivated by phosphorylation and NgCAM is transported to the apical membrane. A series of deletions in the C-terminal cytoplasmic region of NgCAM were fused in frame with the extracellular and transmembrane domains and the first part of the cytoplasmic tail of LDLR to generate chimeras to investigate regions of the distal cytoplasmic tail. Our results indicate that amino acids 45-59 of the NgCAM cytoplasmic tail are sufficient for apical targeting (Fig. 3-4 E). These results support that there is a conserved apical/axonal signal in the NgCAM cytoplasmic tail amino acids 45-59 as this region was sufficient for axonal targeting in hippocampal neurons as well. This region of the tail encompasses the glycine-rich region SASGSGAGSGVGSPG.
Considering that this signal also contains many serine residues, it might be possible that the signal is regulated by phosphorylation. However, Yap et al. saw that mutating all the serine residues to alanines had no effect on polar cell localization in neurons, and therefore, it does not appear that phosphorylation plays a role (Yap et al., 2008). We conclude that the same cytoplasmic sequence in NgCAM promotes both axonal and apical accumulation.

Our data supports work reported in Yap, et al. that tested NgCAM cytoplasmic tail chimeric proteins in hippocampal neurons. Successive truncations of the C-terminus were generated from tail residues 45-114. All the constructs showed similar axonal enrichment. However, the chimera containing residues 66-114 lost axonal enrichment and was easily detected in the dendrites. These results are comparable to basolateral expression of LDLR-CT27+NgCAM 66-114 in MDCK cells (Fig. 3-5 A). However, when mutations are made taking into account known basolateral determinants in MDCK cells, we again see apical enrichment (Fig. 3-5 B-D). Amino acids 66-114 contain a specific ankyrin binding motif, FIGQY 86 and a tyrosine to aspartic acid mutation at amino acid 86 has been shown to disrupt ankyrin B binding (Rubtsov and Lopina, 2000). Binding of NgCAM to ankyrin B was proposed to act as a retention signal. The fact that ankyrin B localizes to axons in neurons but basolateral membrane in MDCK cells might explain why LDLR-CT27+NgCAM 66-114 is non-polarized in neurons, but basolateral in MDCK cells. While cell-types have evolved developing unique cell-specific mechanisms such as the presence of ankryin, it is interesting to investigate these obviously important conserved mechanisms like the signal sequence contained in residues 66-114 of the NgCAM tail that serve as independent axonal/apical sorting information.
MATERIAL AND METHODS

Antibodies

Hybridomas producing anti-LDLR (C7) antibodies were purchased from American Type Culture Collection. Hybridomas producing antibodies directed against gp58 (6.23.3) were generated in the laboratory of Dr. Kai Simons (Max Planck Institute, Dresden, Germany). The 8D9 anti-NgCAM hybridoma was obtained from NIH Hybridoma Bank.

Cell culture

MDCK cells were cultured in MEM containing 7% (v/v) fetal bovine serum, 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin. To allow for polarization, cells were seeded on polycarbonate membrane filters at a density of 4 X 10^5 cells per 12-mm filter (0.4-µm pore size; Corning-Costar Transwell units) and cultured for 3 d with daily changes of the medium in the basolateral chamber.

The filter-grown cells were transiently transfected with cDNAs encoding LDLR-CT27, NgCAM, or LDLR-CT27/NgCAM constructs added to the apical chamber using LipofectAMINE™ (Invitrogen) according to the manufacturer’s protocol and incubated at 37°C for 30 h. For cell surface staining, cells were washed once with ice-cold PBS^2- and incubated with antibodies applied to apical and basolateral sides for 15 min on ice. Cultures were washed 3 X with ice cold PBS^2- and fixed with 3% (w/v) paraformaldehyde (PFA) for 15 min at room temperature. Filters were then cut out and stained for immunofluorescence microscopy essentially as in Chapter 2 and as described previously (Fölsch, 2003).

All immunofluorescence preparations were analyzed using a Zeiss confocal microscope (Microsystem LSM 510; Carl Zeiss MicroImaging, Inc.) with an Axiovert 100 microscope (Carl
Zeiss MicroImaging, Inc.) and a Plan-Apochromat 63X objective. Images were enhanced and combined using Adobe Photoshop®.
CHAPTER 4: GENERAL CONCLUSIONS AND DISCUSSION
During embryogenesis epithelial cells differentiate their plasma membrane into functionally and biochemically distinct plasma membrane domains to form epithelial monolayers. The monolayers are sealed by tight junctions, which form diffusion barriers (Nelson, 2003). It is crucial for polarity maintenance, that the cell correctly sort transmembrane proteins to the correct apical or basolateral membrane throughout their life cycle (Rodriguez-Boulan et al., 2005). This regulation of resident proteins and lipids on these membranes is essential for normal cell function. Loss of cell polarity is a hallmark of metastatic cancer, and investigating how epithelial cells maintain polarity is important for understanding early cancer development. Therefore it is important that we understand the sorting mechanisms of membrane trafficking in the cell.

The trans-Golgi network (TGN) has many regulatory functions in trafficking, and one very important role is that of sorting biosynthetic proteins (Ang et al., 2004; Cancino et al., 2007; Fölsch, 2005). We now understand that recycling endosomes sort biosynthetic or newly synthesized cargo transported from the TGN in addition to endocytosed cargo from the plasma membrane (Ang et al., 2004). This finding is critical because we now have another regulatory mechanism for how new cargo could be moved from the TGN to the plasma membrane to maintain polarity. Previously the scientific dogma was that newly synthesized cargo was only sorted at the TGN but recent studies highlight the recycling endosomes as a destination for many biosynthetic cargos upon which they are sorted to their respective membranes.

We have identified Rab13 as a critical GTPase regulator in this newly observed trafficking pathway from the trans-Golgi network (TGN) to the recycling endosome. In this thesis, we report findings obtained by observations of the co-expression of Rab13 mutants and several different cargo proteins. In the presence of Rab13 mutants, cargos that travel from the
TGN to recycling endosomes were inhibited selectively, indicating the importance of Rab13 in regulating this pathway.

Specifically, we found that mutations of Rab13 resulted in impaired surface delivery of VSVG, A-VSVG, and LDLR-CT27. Furthermore, preliminary data suggest that Rab13 may also regulate surface delivery of LDLR(Y18A/Y35A/Y37A) and mannose-6-phosphate receptor (MPR). Rab13 mutants, however, had little effect upon cargos traveling a direct path to the plasma membrane (LDLR(Y18A), FcR, and HA). These results strongly support the hypothesis that Rab13 is involved in the transport of cargo from the TGN to the recycling endosomes.

Trafficking pathways for numerous receptors and cofactors important for cellular signaling remain to be discovered. Our methods used to determine the function of Rab13 could be used to help determine sorting information for cargo in which the specific transport pathway is not yet established. For example, the sorting of MPR is complex, the protein follows multiple pathways in the cell involving the early, late, and recycling endosomes as well as the plasma membrane (Ghosh et al., 2003a). Our data has already allowed us to suggest that MPRs are biosynthetically sorted to the recycling endosomes before reaching their next cellular destination. In fact, information about cargos and their sorting pathway can be determined using our experimental design, allowing us to predict if the cargos are sorted directly to the membrane or are first sorted to the recycling endosomes during biosynthetic transport.

An important aspect of studying transport pathways regulated by Rab proteins is to determine which organelles the Rab proteins are involved with for inter-membrane transport. Due to the vesicular transport function of Rab proteins in the cell between membrane domains, Rab proteins are known to localize to both their donor and acceptor organelles and therefore likely function as gatekeeper molecules escorting cargo in vesicular transport to and from
membranes to maintain proper localization (Collins, 2003). Our findings report localization of Rab13 at the TGN and recycling endosomes and supports the hypothesis that Rab13 functions at the interface of these two compartments in the cell. Previously Rab13 has been shown to play a role in tight junction integrity and maintenance. However, previous data collected in other labs are based on prolonged overexpression of Rab13 mutants or were based on experiments performed in a stable cell line expressing GFP-tagged Rab13. As we show here, longer incubation protocols lead to a disruption of the Golgi complex. Therefore our experimental design using microinjection and expression of Rab mutants over a shorter time frame best describes the overall role of Rab13. Based on our experiments we demonstrated a distinct phenotype after shorter expression times, and this may in fact be more reflective of the role of Rab13 in the cell, since we do not yet observe secondary effects such as tight junction and Golgi phenotypes. Our findings are also strengthened with the appearance of a phenotype for both the dominant active and dominant negative Rab 13 mutant.

Rab family members generally have a role in regulating vesicular trafficking. The Rab family of GTP-binding proteins has been classified into subfamilies according to conserved sequence patterns. Pereira-Leal and Seabra used phylogenetic analysis to reconstruct the evolution of the Rab family. So far there are over 60 Rab proteins identified in mammalian cells. The subfamilies were based on conserved sequence patterns and they hypothesize that the family shows a strict phylogeny of function as opposed to a phylogeny of species, meaning they observed Rabs co-segregated in phylogenetic trees showing a pattern of similar cellular localization and/or function (Pereira-Leal and Seabra, 2001). Rab8, Rab10, and Rab13 are grouped into the same subfamily and are among the closest mammalian homologues of Sec4p. In yeast, Sec4p is known to regulate polarized exocytic transport to the emerging bud tip (Novick
et al., 1988). Specificity of Rab proteins is often encoded in the hypervariable C-terminus of the protein. The majority of Rab proteins are modified post-translationally by two 20-carbon geranylgeranyl groups that are linked covalently to cysteine residues at their C-termini, usually associated with a di-cysteine motif such as CC or CXC. In the case of Rab8 and Rab13 the C-termini are isoprenylated, modified by a single geranylgeranyl moiety at the CAAX motif (Joberty et al., 1993). Prenylation is necessary for two facets of Rab function. First, it is required for insertion of the GTP-bound Rab into the membrane. Second, it promotes optimal interaction of the GDP-bound form of the Rab protein with GDI wrapped in a hydrophobic pocket.

Previously, Rab8 was reported to regulate basolateral sorting from the recycling endosomes to the plasma membrane (Ang et al., 2003; Au et al., 2007). The data for Rab10 are more convoluted, however, it is apparent that this GTPase is involved in endosomal transport of cargo. We suggest that the co-segregation into the phylogenetic subfamily for Rab8, Rab10, and Rab13 not only reflect a pattern for sequence similarity but also show a pattern of similar cellular function not before realized. These three GTPases are all responsible for transport in or out of the recycling endosomes. However, we also show that Rab13 has its own signaling properties. Therefore we identified an evolutionary purpose for multiple Rab proteins that have some overlapping and some distinct functions.

Rab 8, Rab10, and Rab13 share strong amino acid sequence homology. Therefore, to establish a unique function for Rab13 it was important to compare Rab13 with Rab8 and Rab10 in detail. This was accomplished with the experiments carried out in Chapter 2 of this thesis. In Fig. 2-8 and Fig. 2-10 we analyzed cells that were co-injected with dominant active or dominant negative mutant forms of Rab8, Rab10, and Rab13 and TGN38 cDNA into MDCK cells grown on coverslips. Displacement of TGN38 from the TGN was specific for the Rab13 constructs.
Therefore, despite similar amino acid sequences and similar isoprenyl groups, the function of Rab13 remains different than that of Rab8 and Rab10. Future studies could utilize dissimilar regions of the Rab proteins to begin to understand how they function differently. Already, we obtained yeast two-hybrid data that suggest at least two interesting binding partners. Based on these interactions, we can design site-directed mutagenesis on Rab13 to unravel more specific molecular interactions.

Our lab focuses on utilizing polarized epithelial cells derived from dog kidneys as a model system. However, in this study, we confirmed Rab13’s effects on the TGN in another physiologically relevant system, the human bronchial epithelial cell line 16HBE14o- (HBE). Using RNAi we knocked down Rab13 expression in the cell, which resulted in the disruption of the TGN46 (primate homologue of TGN38). Interestingly, this was the same change that we saw in the MDCK line transfected with the mutants designed to block signaling, confirming the need for Rab13 in TGN sorting. We conclude that Rab13 is essential for organizing subdomains of the TGN and subsequent transmembrane protein delivery. The TGN is a dynamic structure at the intersection of endocytosis and exocytosis. This organelle therefore requires resident integral membrane proteins to help maintain TGN integrity, including putative cargo binding proteins such as TGN38/46 (Ponnambalam and Banting, 1996). Other proteins thought to be important for TGN integrity are the golgin tethers including, golgin-245, golgin-97, GCC88, and GCC185. In addition to disruption of TGN46, preliminary results suggest that the knock down of Rab13 in HBE also leads to a disruption of golgin-97. The TGN golgins are large peripheral membrane proteins characterized by extensive coiled-coil structure and a conserved C-terminal GRIP (Golgin-97, RanBP2α, Imh1p and trans golgi p230) domain. The GRIP domain confers localization to the TGN. Burguete et al. reported that the recruitment of GCC185 to the Golgi is
mediated by the binding of Rab6. The crystal structure of Rab6 bound to the GCC185 Rab-
-binding domain reveals that Rab6 recognizes a coiled-coil domain immediately adjacent to a C-
terminal GRIP domain and Rab6 binding promoted association of Arl1 with the GRIP domain
(Burguete et al., 2008). Arl1 is a member of the ADP-ribosylation factor (Arf) subfamily of the
Ras superfamily and is necessary for TGN maintenance (Lu et al., 2001). In the future it will be
interesting to test if the knock down of Rab13 in HBE cells also disrupts the other golgin family
member proteins. Regardless of whether the effect of Rab13 knock down disrupts only golgin-
97 or multiple golgin proteins, it is interesting to further investigate possible interactions of
Rab13 with other proteins functioning at the TGN. In addition to RNAi experiments, other
suggested experiments include anti-Rab13 co-immunoprecipitation or in vitro pull down assays
with His-tagged Rab13 to analyze whether any of the TGN golgins directly interact with Rab13.

PDEδ was isolated as the δ–subunit of the rod-specific phosphodiesterase, but was later
shown to be the structural homologue of guanine nucleotide dissociation inhibitor (GDI), able to
extract prenylated proteins such as Rab13 from membranes (Marzesco et al., 1998). PDEδ is
widely expressed and highly conserved across evolution. As well as extracting Rab13 from the
membrane, PDEδ also regulates the catalytic subunits of the PDE holoenzyme involved in
phototransduction. Other proteins known to interact with PDEδ include the retinitis pigmentosa
GTPase regulator (RPGR). RPGR is the gene product of RP3 (retinitis pigmentosa gene 3) and
mutations in this gene are the major cause of XLPR (retinitis pigmentosa with an X-linked
hereditary pattern) (Linari et al., 1999b). Retinitis pigmentosa refers to a heterogeneous group of
inherited ocular diseases that result in retinal degeneration and XLPR is the most severe form of
the disease. PDEδ is believed to act as the GDI for Arf-like protein, Arl3, another small GTPase
(Linari et al., 1999a) and recently the retinitis pigmentosa 2 (RP2) gene product was identified as
an efficient GTPase-activating protein (GAP) for Arl3 (Veltel et al., 2008). This thesis confirms by yeast two-hybrid the interactions previously described between Rab13 and PDEδ. We have reported that Rab13 does play a role at the TGN previously never described. In the future it will be interesting to further elucidate the molecular mechanisms of Rab13 and investigate possible interactions Rab13 may have with proteins such as Arl1 and Arl3 in addition to TGN golgins that are associated with the TGN and might result in ocular disease when mutated.

We suggest that the results found before for Rab13 were due to secondary effects and that in fact Rab13’s primary function is for transport in the novel pathway from the TGN to recycling endosomes. The key to this discovery was using the technique of microinjection as a valuable tool for experimentation. In addition, we showed that RNAi are suitable for analyzing Rab13’s primary function.

In Chapter 3, transmembrane cargo proteins were analyzed for conserved signaling information. We find that the same cytoplasmic sequences in NgCAM promote both axonal/neuronal as well as apical/epithelial cell accumulation. Elucidation of the molecular mechanisms behind protein trafficking routes and sorting machinery is fundamental to understanding how cells establish and maintain polarity and therefore to understanding disease-related illnesses in which cell have lost their ability to form healthy, polarized cells.

In summary, this thesis established Rab13 as the first player in the trafficking pathway from the TGN to the recycling endosomes. Future research may now focus on mechanistic analysis and how Rab13 is functioning in this transport.
APPENDIX I: SUPPORTING INFORMATION
INTRODUCTION

In our investigation of Rab13 and its role at the trans-Golgi network (TGN) and recycling endosomes, some additional lines of investigation not discussed in Chapter 2 led to results that are interesting and contribute to the larger picture in determining the function of Rab13. This section includes the observations from several experiments that may prove important to the field in the coming years, including an analysis of Rab13 protein interactions from yeast two-hybrid studies, and the surface localization of other cargo proteins affected by Rab13.

Rab13 is critical for TGN sorting of cargo proteins and therefore important for cell polarity maintenance. We therefore attempted to identify proteins that interact with Rab13 to unravel the players in the cell that assist in Rab13 mediated protein sorting. A yeast two-hybrid screen can help to identify possible protein-protein interactions. We had the company Proteinlinks, Inc. perform a yeast two-hybrid screen against the fetal human kidney cDNA library as the ‘prey’ of the screen and the dominant active mutant Rab13Q67LΔC as the ‘bait’. The screen reported one positive interaction between the C-terminal fragment of the 20S proteasomal α-subunit, Psma7 and Rab13Q67L.

We next tested specific yeast two-hybrid interactions of Rab13 with Psma7 identified in the screen. In addition, we tested for interactions of Rab13 and PDEδ, previously reported to have an interaction with Rab13. Rab13 wild-type and mutant proteins were shown to intact with PDEδ (Marzesco et al., 1998). It is reported that PDEδ is capable of extracting Rab13-GDP from the membrane and therefore acts as the GDP-dissociation inhibitor (GDI) for Rab13 instead of the traditional Rab guanine nucleotide dissociation inhibitor (GDI), RabGDI (Nancy et al., 2002).
The yeast two-hybrid assay requires the expression of bait and prey proteins coming together to stimulate transcription of an indicator gene (Fields and Sternglanz, 1994). In this study, we used Psma7 and PDEδ as our bait and Rab13 and Rab8 wild-type (WT) and mutant constructs as our prey. We chose the C-terminus of Psma7 (Psma7ΔN) since it was identified in the screen of Rab13Q67LΔC and the human kidney cDNA library. In addition we tested the full length Psma7. We used both the full length and truncated versions of the Psma7 protein due to a possible steric impediment caused by the N-terminus of this protein. PDEδ was chosen because of its previously mentioned interaction with Rab13. We found the strongest interactions (as determined by growth on 1 mM 3-AT/TDO plates) between both Rab13WT and the dominant negative Rab13T22N mutant and Psma7ΔN.

LDLR is a useful tool for analyzing membrane trafficking because its cytoplasmic tail contains two basolateral sorting determinants and it has been investigated in detail. (Matter et al., 1992) (see Figure A-1). The proximal signal located closer to the transmembrane domain is a FXNPXY motif (residues 13-18) and is important for sorting of the cargo through recycling endosomes (Bonifacino and Traub, 2003; Fields et al., 2007). Tyrosine 18 has also been described as being involved in endocytosis and is thought to interact with AP-2 through co-adaptor proteins, numb, Dab2, and ARH (Maurer and Cooper, 2006). Although direct interactions with μ1B of the AP-1B adaptor complex have not been found, tyrosine 18 is functionally dependent on the presence of μ1B (AP-1B) for basolateral sorting. The LDLR C-terminal truncation mutant (LDLR-CT27) removes the distal basolateral determinant and therefore its basolateral targeting is dependent on only the tyrosine 18 proximal sorting signal. LDLR-CT27 is sorted as biosynthetic cargo first to the recycling endosomes and then is sorted in an AP-1B-dependent manner to the basolateral membrane (Fields et al., 2007). The distal
The cytoplasmic tail of LDLR contains two basolateral sorting determinant (Matter et al., 1992). The proximal signal is dependent on tyrosine 18 and is necessary for biosynthetic transport to the recycling endosomes. The proximal signal has been shown to interact with AP-2 via the co-adaptors, numb, Dab2, and ARH (Maurer and Cooper, 2006). Although a direct interaction between this signal and µ1B (AP-1B) cannot be established, the correct sorting of LDR-CT27 requires this tyrosine and the presence of µ1B for sorting to the basolateral membrane in polarized MDCK cells. The distal non-canonical tyrosine signal has shown yeast two-hybrid interactions with µ2 (AP-2) and µ4 (AP-4) (Fields et al., 2007).
targeting determinant is considered to be non-canonical and is AP-1B-independent. In sorting LDLR(Y18A), the GYSY sorting motif is the active basolateral sorting signal interacting with adaptor proteins in transport and it is sorted from the TGN directly to the basolateral membrane perhaps involving AP-4 (Fields et al., 2007). Mutation of tyrosine to alanine at residues 18, 35, and 37 [LDLR(Y18A/Y35A/Y37A)] results in the loss of both proximal and distal basolateral sorting determinants and LDLR(Y18A/Y35A/Y37A) is therefore largely expressed at the apical membrane in MDCK cells. Analysis of this third LDLR mutant LDLR(Y18A/Y35A/Y37A) gives us additional information to support Rab13’s role at the level of the TGN and recycling endosomes.

Another cargo protein we investigated was the mannose 6-phosphate receptor (MPR). The sorting of MPR and its association with the endosomes has been well studied. MPRs include the ~46 kDa cation-dependent MPR (CD-MPR) and the ~300 kDa cation-independent MPR (MPR). The cation-independent MPR is a multifunctional receptor that is involved in many tasks essential for cellular function. MPRs bind acid hydrolases in the TGN and are packaged into transport carriers that deliver the receptor with its bound ligand to the early endosome (Ghosh et al., 2003a). MPRs travel many pathways in the cell and after entry into early endosomes can be sorted to the late or recycling endosomes. Upon arrival at the basolateral membrane, MPRs are endocytosed in clathrin-coated vesicles (CCVs) containing AP-2. MPR is a promiscuous cargo and has been associated with multiple adaptor proteins at the TGN and early endosomes, including AP-1, the Golgi-localized, γ-ear-containing, adenosine diphosphate ribosylation factor binding proteins (GGAs), and phosphofurin acidic cluster sorting protein (PACS-1). MPRs are localized to AP-1A CCVs at the TGN. Doray et al. report that the GGAs function as adaptor proteins that select MPR molecules for incorporation into AP-1A
containing CCVs (Doray et al., 2002). The GGAs are a family of multidomain proteins in mammalian cells, numbered 1-3. The same group later reported that in fact all three family members cooperate to sort cargo, specifically MPR, and GGAs 1-3 are required for maintenance of TGN structure (Ghosh et al., 2003b). One study finds that a complex of PACS-1 and GGA3 regulate the trafficking of MPR (Scott et al., 2006). Regardless, it is obvious that MPR is capable of multiple trafficking pathways. We tested the effect of overexpressed Rab13 mutants on the surface arrival of MPR.

RESULTS

Yeast two-hybrid screen

In an attempt to identify effector proteins of Rab13 we had the company Proteinlinks, Inc. perform a yeast two-hybrid screen against the fetal human kidney cDNA library as the ‘prey’ of our screen. We hypothesized that because the MDCK cells are derived from the kidney, Rab13 associated proteins could be found in an analogous human system. We used the dominant active mutant Rab13Q67LΔC as ‘bait’ because the active conformation is involved in binding to its effector proteins. Furthermore, the deletion of the prenylation signal of the C-terminus often stabilizes Rab proteins in yeast two-hybrid assays. Two possible candidates (#1 and #6) were identified (Fig. A-2). In fact, the interacting clones were found to be transcripts for the C-terminal fragment of the 20S proteasomal α-subunit, Psma7. Although the interaction of Psma7 and Rab13 has not been published, this screen did not identify a Rab13 interaction with the rod cGMP phosphodiesterase δ subunit (PDEδ), an interaction that has been previously identified most likely because interaction between Rab13 and PDEδ requires isoprenylation
Figure A-2: Yeast two-hybrid screen shows two possible interacting proteins for Rab13Q67LΔC.

The active Rab13 mutant was screened against 0.6 x 10^7 cDNA clones from the library. After selection for URA3^+ there were 33 preliminary clones. To verify interacting clones possible positives were streaked out on X-gal/glucose plates (X-Gal/Glu). A successful interaction is translated into reporter gene activity of X-gal and the colonies turn bright blue on X-gal/galactose plates (X-Gal/Gal). Two possible candidates (#1 and #6) were identified. Positive control interactions can be observed with the p53(50-363) as bait and with Pias 1 as a prey.
of Rab13 at the C-terminus (Marzesco et al., 1998).

**Testing yeast two-hybrid interactions**

The results reported from the yeast two-hybrid screen prompted us to further investigate the yeast two-hybrid interactions of Rab13 with Psma7 identified in the screen. In addition, we tested for interactions of Rab13 and PDEδ. Previously, wild-type and mutant Rab13 proteins were shown to interact with PDEδ (Marzesco et al., 1998). To investigate possible yeast two-hybrid interactions we used Psma7 and PDEδ as our bait and wild-type (WT) and mutant Rab13 and Rab8 as our prey in this assay. For this yeast two-hybrid system, growth on cell medium lacking histidine suggests an interaction between bait and prey proteins. This means that yeast cells are able to grow on triple dropout plates (TDO), which lack tryptophan, leucine, and histidine. The enzymes needed to grow on plates missing tryptophan and leucine is encoded in the corresponding bait and prey plasmids. Yeast colonies are then streaked out on TDO plates plus 1 mM 3-amino-1,2,4-triazole (3-AT). The presence of 3-AT should inhibit growth of colonies that only have a leaky expression of the reporter gene because 3-AT inhibits histidine biosynthesis.

The ‘bait’ proteins were fused to the GAL4 DNA-binding domain in pAS2-1 plasmids. The bait proteins tested included Rab13 constructs, Rab13WT, Rab13Q67L, Rab13T22N, and Rab13Q67L/G113D. In addition we tested Rab8 constructs as bait proteins including, Rab8Q67L and Rab8T22N the GTP and GDP-bound forms as well as Rab8WT. Rab8 is among one of the closest homologues of Rab13. Therefore, Rab8 proteins were used for testing the promiscuity of the interactions.
The ‘prey’ proteins tested were fused to the GAL4 activation domain in the pACT-2 construct. Interaction between the bait and prey proteins brings both GAL4 fragments together and drives the expression of a reporter protein, in this case an enzyme in the histidine biosynthetic pathway. The yeast two-hybrid screen performed by Proteinlinks, Inc. found an interaction with Rab13-GTP and a C-terminal truncation of Psma7. When testing the yeast two-hybrid interactions we tested the truncated version of Psma7 as well as the full-length protein. We also tested for interactions between Rab8 or Rab13 constructs and PDEδ.

After transformation of the yeast, all plates yielding six or more colonies were streaked out first on DDO plates and then TDO plates. PDEδ appears to interact with Rab13Q67L and Rab13Q67L/G113D. All forms of Rab13 appear to interact with Psma7ΔN as indicated by growth on TDO plates. It is important to note, that no interaction of Psma7 with any form of Rab8 was observed. Yeast colonies were then streaked on 3-AT TDO plates to detect stronger protein interactions between bait and prey. The interactions of Rab13WT and Rab13T22N with Psma7ΔN are the strongest interactions as judged by growth on 3-AT TDO plates (Fig. A-3). The other interactions (PDEδ with Rab13Q67L and Rab13Q67L/G133D, as well as Psma7ΔN with Rab13Q67L and Rab13Q67L/G113D) were not capable of rescuing growth in the presence of 3-AT.

Rab13Q67L/G113D has an additional mutation in the Rab complementary determining region (CDR). The CDR is thought to be the structural basis for Rab effector specificity (Ostermeier and Brunger, 1999). We expect that mutating the Rab CDR domain in the
Figure A-3: Rab13WT and dominant negative mutant, Rab13T22N show the strongest interaction with Psma7ΔN as judged by yeast growth on 1 mM 3-AT TDO plates.

Yeast strain AH109 was co-transformed with plasmids expressing Rab8 or Rab10 constructs and Psma7ΔN. The co-transformants were then streaked out on DDO, TDO, and 1 mM 3-AT TDO plates. Images shown are yeast colonies on 1 mM 3-AT TDO plates representing the strongest protein-protein interactions.
Rab13Q67L/G113D mutant will inactivate any GTP specific interaction. Importantly, no protein interactions were seen for Rab8 and Psma7 demonstrating the specificity for these proteins as Rab13 effectors. Yeast two-hybrid interaction results are summarized in Table A-1.

Generation of a polyclonal Rab13 and Rab8 Antibodies

Many of our investigations into Rab13’s function were hampered by the lack of a specific antibody for that protein. Because of this, we have chosen to use cloning strategies that incorporate sequences for known epitope tags into expression constructs. While epitope tags are designed to have minimal impact upon protein production, incorporation of such tags might alter protein folding and change localization signals. Therefore we attempted to design our own antibody to detect Rab13. Polyclonal rabbit antibodies were raised against a Rab13 synthetic peptide sequence, Rab13 C-terminus C-NKPPSTDLDKTDKNT, and generated by Genemed Synthesis, Inc. In addition we had antibodies made against synthetic peptide sequence, Rab8 C-terminus C-SNQGVKITPDQQKRSSF for detection of Rab8WT and mutant alleles.

Genemed Synthesis, Inc. provided us with affinity purified antibodies and the remaining flow-through antisera. MDCK and LLC-PK1 (porcine kidney epithelial) cell lysate (1µg/µL) was resolved by SDS-PAGE. Affinity purified antibody of several dilutions, and flow through from the purification protocol, were used to detect Rab13. Unfortunately, there was no specific binding and the Rab13 polyclonal antibody is unusable for detection in western blot analysis (data not shown).
<table>
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<th>Rab13WT</th>
<th>PDE6D (TDO)</th>
<th>PDE6D (3AT/TDO)</th>
<th>Psma7ΔN (TDO)</th>
<th>Psma7ΔN (3AT/TDO)</th>
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Table A-1: Summary of Rab13 yeast two-hybrid interactions.
Protein pairs able to rescue growth on TDO (triple drop out plates; lack tryptophan, leucine, and histidine) are designated as black ‘*’. Psma7 and Rab13WT and Rab13T22N protein pairs showed the strongest interactions as representative of growth on 1 mM 3-AT containing TDO plates. These interactions are designated as red ‘*’.
The affinity purified Rab13 polyclonal antibody was also used in experiments to test Rab13 localization in epithelial cells. MDCK cells were grown on coverslips and processed for immunofluorescence. Endogenous levels of Rab13 are too low for detection using Rab13 polyclonal antibody. The antibody was able to detect transiently transfected Rab13Q67L in the perinuclear region (Fig. A-4). We were unable to use this antibody for detection of Rab13T22N (data not shown). This is not surprising since the antibody is generated against the hypervariable C-terminus that is most often buried in the GDI/PDEδ when Rab13 is in the cytosol. In any case, detection using this antibody proved to be below the quality of experimental results achieved using the V5-tag on Rab13 for detection.

Overexpression of Rab13 mutants has no discernable effect on AP-3 localization

As discussed in Chapter 2, overexpression of Rab13 mutants by microinjection disrupted localization of the TGN marker, TGN38 (Fig. 2-8). This effect was specific for TGN38 because microinjection of Rab13 mutants had no discernible effect on the localization of GM130, γ-adaptin (AP-1A and AP-1B), or TfnR (Fig. 2-11). In addition we looked for disruption of another adaptor protein sometimes associated with the TGN, AP-3. Preliminary results suggest that overexpression of Rab13 mutants by microinjection has no effect on the localization of AP-3 (Figure A-5).
Figure A-4: Testing of polyclonal Rab13 antibody.
MDCK cells seeded on coverslips were transiently transfected with cDNAs encoding V5-Rab13Q67L) or V5-Rab12T22N. Cells were incubated at 37°C for 24 hr. Subsequently, cells were fixed and immunolabeled for Rab13 using either the polyclonal Rab13 antibody or anti-V5 antibodies followed by an incubation with the following secondary antibodies: goat anti-rabbit Alexa 488 or goat anti-mouse Alexa 594 (V5-Rab13). Specimens were analyzed by confocal microscopy and representative XY images are shown.
Figure A-5: Overexpression of Rab13 mutants by microinjection has no discernable effect on AP-3 localization.

MDCK cells grown on coverslips were microinjected with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N and incubated at 39°C and 31°C as described for VSVG in Fig. 2-2. Cells were then fixed and processed for immunofluorescence staining for V5-Rab13 (anti-V5 tag antibodies [mouse IgG2a]) and AP-3 (ASA4[mouse IgG1]). Subsequently, cells were incubated with isotype specific, fluorescently labeled secondary antibodies. Specimens were analyzed by confocal microscopy and representative XY images are shown. Scale bars are 10 μm.
Rab13 nucleotide-free mutant

Our investigations found that overexpression of a dominant active and a dominant negative form of Rab13 gave the same overall results in terms of sorting from the TGN. We therefore attempted to generate another type of dominant negative mutant to test whether this inactive form would be equally effective. We generated a Rab13 point mutant, Rab13D124A. This mutation replaces a charged amino acid with a hydrophobic amino acid in the C-terminus of Rab13. This mutant protein is unable to bind nucleotides and therefore is thought to act as a dominant negative (Dong et al., 2004). In the future this construct may be used to analyze if in fact it does induce a phenotype similar to Rab13T22N when used in experiments.

Using dominant negative Rab13 to analyze cargo sorting pathways

In Chapter 2, we demonstrated that surface delivery of LDLR-CT27 protein is inhibited with the co-expression of Rab13 mutants. As previously discussed, LDLR-CT27 is sorted to the basolateral membrane in an AP-1B-dependent pathway similar to VSVG (pathways 3 and 3b in Fig. 2-5 A). We also analyzed the surface arrival of LDLR(Y18A) that is thought to travel directly from the TGN to the basolateral surface independent of AP-1B function (Fields et al., 2007; Roush et al., 1998). As shown in Fig. 2-5 C, the overexpression of Rab13 mutants had no effect on surface delivery of LDLR(Y18A).

In addition to the LDLR constructs analyzed in Chapter 2, we also investigated the surface arrival of LDLR(Y18A/Y35A/Y37A). The triple tyrosine to alanine mutant, LDLR(Y18A/Y35A/Y37A) is mutated so that both proximal and distal basolateral sorting signals are lost and therefore the protein is mislocalized to the apical membrane. This mutant
LDLR construct was co-injected with Rab13 mutant cDNA and the surface arrival of cargo was judged by surface staining (experimental details reviewed in Chapter 2). Upon co-expression of the Rab13 mutant cDNA constructs, apical delivery of LDLR(Y18A/Y35A/Y37A) was severely inhibited (Fig. A-6 A). Therefore, our results seem to suggest that the LDLR mutant protein, LDLR(Y18A/Y35A/Y37A) is transported from the TGN to the apical membrane via recycling endosomes.

We also tested the delivery of mannose 6-phosphate receptor (MPR) in the presence of Rab13 mutant alleles to understand if Rab13 alters MPR surface arrival. V5-tagged Rab13Q67L and Rab13T22N cDNAs were microinjected into filter-grown, polarized, MDCK cells. Upon co-expression of the Rab13 mutants we observed severe inhibition of surface delivery of MPR to the basolateral surface (Fig. A-6 B). Our findings suggest that perhaps MPR might traffic through the recycling endosomes during biosynthetic delivery to the surface.

Our studies showed that Rab13 has a role in transport between the TGN and recycling endosomes. Insight into the transport of cargo proteins can now be gained by reporting surface arrival results of cargo when co-expressed with Rab13 mutants. We hypothesize that cargo in which Rab13 inhibits surface delivery are transported through the recycling endosomes in route to their respective plasma membranes.

Depletion of Rab13 by RNAi in human bronchial epithelial cells leads to disruption of golgin-97

In Chapter 2, we investigated the effects of Rab13 in a physiological system by inducing the knock down of Rab13 in the human bronchial epithelial cell line 16 HBE14o- (HBE). Three GFP-tagged shRNA constructs targeting Rab13 and one control construct targeting GAPDH
Figure A-6: Rab13 mutant expression disrupts surface arrival of LDLR mutant protein LDLR(Y18A/Y35A/Y37A) and MPR.

(A-B) Fully polarized MDCK cells were co-injected with cDNAs encoding V5-Rab13Q67L or V5-Rab13T22N and cDNAs encoding LDLR mutant protein LDLR(Y18A/Y35A/Y37A) (A) or myc-MPR (B). Injected cells were incubated for 1 h at 37˚C followed by 4 h at 20˚C and a 2 h chase at 37˚C in the presence of CHX. Surface staining for anti-LDLR antibodies (C7) or myc-MPR was followed by cell permeabilization and staining for V5-Rab13 with anti-V5 antibodies. Cells were incubated with fluorescently labeled secondary antibodies: Alexa 594-labeled secondary goat anti-mouse IgG2b antibodies (A) or goat anti-rabbit Alexa 594 (B) and goat anti-mouse IgG2a, Cy5(V5-Rab13). Specimens were analyzed by confocal microscopy and representative XZ images are shown.
were obtained from Open Biosystems. As mentioned previously, we found that constructs Rab13 #1 and Rab13 #3 knocked down expression (Fig. 2-14 A), whereas construct #2 was ineffective (unpublished observation). In addition to experiments describe in Chapter 2, the constructs were transfected into HBE cells and transfected cells were stained with antibodies against TGN46 and golgin-97 48 h post-transfection. We found that knock down of Rab13 resulted in disruption of golgin-97 (Fig. A-7). These data, in addition to the results observed in Chapter 2, indicate that Rab13 is needed for the maintenance of a TGN subdomain defined by TGN38/TGN46 and golgin-97 localization.

**DISCUSSION**

In Chapter 2 we reported results for the co-expression of Rab13 mutants and several different cargo proteins. In summary, we found that cargos that biosynthetically sort through recycling endosomes (VSVG, LDLR-CT27, and A-VSVG) are inhibited for surface delivery to their respective membrane domains when co-expressed with Rab13 mutants. Additionally, Rab13 mutants had little effect upon cargos traveling a direct path to the plasma membrane [LDLR(Y18A), FcR, and HA]. As shown in Figure A-6, the surface arrival of MPR and LDLR(Y18A/Y35A/Y37A) is inhibited upon co-expression of Rab13 mutants. These results suggest that these cargos are biosynthetically sorted from the TGN to the recycling endosomes.

This chapter also addresses possible protein-protein interactions involving Rab13 as determined by yeast two-hybrid assays. To further our understanding of Rab13 and its effector proteins, a yeast two-hybrid screen was performed with a human kidney cDNA library. This screen resulted in the identification of one candidate interaction protein, Psma7. Previously
Figure A-7: Knock down of Rab13 in HBE cells leads to a disruption of TGN46 and golgin-97 localization.

HBE cells were seeded onto coated coverslips and transfected with plasmids encoding shRNA constructs (upper panel GAPDH, middle panel Rab13 #1, and bottom panel Rab13 #3). 48 h post-transfection, cells were fixed and labeled with anti-TGN46 antibodies (in red, using goat anti-rabbit Alexa 594-labeled secondary antibodies) and anti-golgin-97 antibodies (in blue, using goat anti-mouse Cy5-labeled secondary antibodies). Specimens were analyzed by confocal microscopy and representative images are shown. Scale bars are 10 µm.
Psma7 was shown to have a protein interaction with another Rab protein, Rab7. Psma7 is thought to be recruited to the late endosomes through this interaction (Dong et al., 2004). We confirmed the Rab13 and Psma7 interaction in yeast two-hybrid assays performed in our laboratory. This interaction is specific for Rab13 as compared to Rab8; however, nothing else is known about this possible interaction or its actions in vivo.

Previously the wild-type as well as mutant Rab13 proteins were shown to interact with PDEδ (Marzesco et al., 1998). It is suggested that PDEδ acts as the GDI for Rab13 and is capable of extracting Rab13-GDP from the membrane (Nancy et al., 2002). PDEδ interacts with the Arf-like protein, Arl3, and Arl3 recruits Arl1 to the TGN, and Arl1 is necessary for TGN maintenance (Linari et al., 1999a; Lu et al., 2001; Panic et al., 2003). In the future it will be interesting to further understand Rab13’s role at the TGN and the possible interaction of Rab13 and other TGN maintenance proteins.

In this study, a depletion of Rab13 by RNAi in the human bronchial epithelial cell line (HBE) led to a disruption of Golgi localization of the TGN38 homologue, TGN46. In addition, a TGN-resident protein of the golgin family of proteins involved in organizing the Golgi and TGN, golgin-97, was also disrupted upon knock down of Rab13 (Fig. A-7). In the future it will be interesting to monitor the localization of the TGN golgins, golgin-97, golgin-245, GCC88 and GCC185 in HBE cells treated with Rab13 RNAi. Typically, the TGN golgins are important for retrieving TGN resident proteins back to the TGN. Interestingly, golgins have been described as Rab effectors. For example, Rab9 has been shown to interact biochemically with GCC185 and Rab6 interacts with golgin-97 and golgin-245 (Burguete et al., 2008; Yoshino et al., 2003). Using biochemical analysis, it would be interesting to analyze whether Rab13 interacts directly with any of the TGN golgins. Having established a role for Rab13 at the interface between TGN
and recycling endosomes, we can now begin to analyze Rab13’s interplay with known regulators of the TGN.

MATERIAL AND METHODS

Cloning for yeast two-hybrid analysis

The yeast two-hybrid system used in this study has been already described (Fields et al., 2007; Wallach et al., 1998). Briefly, pCMV6-XL5 plasmids with the genes for the bait proteins Psma7 and PDE6D (PDEδ) were purchased from Origene. The bait constructs (Psma7, Psma7ΔN and PDE6D) were subcloned into the pAS2-1 vector (Clonetech Laboratories, Inc) and the prey constructs, the Rab8 and Rab13 mutants were subcloned into the pACT-1 vector (Clonetech Laboratories, Inc). Primers containing restriction sites (BamHI or EcoRI) were used to amplify DNA from the original plasmids. Two N-terminal primers were used to amplify the Psma7 gene. The ‘N-primer’ (listed below), anneals and amplifies the full-length protein. The ‘NΔ-primer’ anneals 522 base pairs downstream of the start codon, and the PCR product encodes a truncated protein lacking 173 N-terminal residues. The primers used are listed below:

Rab8 N-primer: 5’ GCGCGAATTCATGGCGAAGACCTACGATTAC 3’
Rab8 C-primer: 5’ GCGCGGATCCTCAGAAGAACATCGG 3’
Rab13 N-primer: 5’ GCGCGAATTCTCATGCGAAGACCTACGATTAC 3’
Rab13 C-primer: 5’ GCGCGGATCCTCAGCCCAGGGAGCACTTGTTGTGTT 3’
PDE6D N-primer: 5’ GCGCGGATCCGAGCTACGACCGCCGAGGACGACGCGG 3’
PDE6D C-primer: 5’ GCGCGAATTCTCAAGGAGGAGGAGGACGACGCGG 3’
Psma7 N-primer: 5’ GCGCGGATCCGAGCTACGACCGCCGAGGACGACGCGG 3’
Psma7 NΔ-primer: 5’ GCGCGATCCGAAGAAACTATACTGACGAAGCC 3’
Psma7 NΔ-primer: 5’ GCGCGAATTCTCATGATGCTTTCTTTTGTTC 3’
PCR products and vectors were digested with BamHI and EcoRI restriction endonucleases, purified and ligated. TOP10F Escherichia coli were transformed and clones were selected according to test digest results of miniprep DNA for use in the yeast two-hybrid analysis.

Yeast two-hybrid screen

The dominant active mutant Rab13Q67LΔC, the ‘bait’, was subcloned into the pCWX200 plasmid and verified through sequencing. The screen was performed by ProteinLinks, Inc. The active Rab13 mutant was screened against 0.6 x 10⁷ cDNA clones from the library. In general, yeast colonies with positive binding domain interactions express the URA3 gene required for uracil synthesis. URA3⁺ selection identified 33 preliminary clones. To verify interacting clones, possible positive clones were streaked out on X-gal/galactose plates. A successful interaction is translated into reporter gene activity of X-gal and the colonies turn bright blue.

Yeast two-hybrid assays

Twenty-one pairs of DNA constructs were co-transformed in yeast, each pair corresponding to one of the seven bait proteins (Rab8WT, Rab8Q67L, Rab8T22N, Rab13WT, Rab13Q67L, Rab13T22N and Rab13Q67L/G113D) and one of three prey proteins (PDEδ, Psma7 and Psma7ΔN). Empty plasmids were used as negative controls. Lithium acetate co-transformations were performed using the Saccharomyces cerevisiae AH109 strain. The co-
transformed yeast cells were plated on selective medium lacking tryptophan and leucine (DDO), and incubated at 30°C for six to seven days. Six colonies, of similar size when possible, were picked from each plate and streaked onto new DDO plates and incubated for three days at 30°C. One clone from each protein pair was then streaked onto TDO plates (lacking tryptophan, leucine and histidine) and incubated for three to four days at 30°C. Growth of yeast in medium lacking histidine indicates an interaction between the bait and prey proteins, containing the GAL4 DNA-binding and GAL4 activation domain. Clones from DDO plates were also streaked out on 1 mM 3-AT TDO plates and incubated for three days at 30°C. This should inhibit some growth of colonies that show weak interactions between the prey and the bait proteins.

**Generation of Rab13 and Rab8 Antibodies**

Polyclonal antibodies were raised against Rab13 or Rab8 synthetic peptide sequence and generated by Genemed Synthesis, Inc. The peptide sequences were derived from the C-termini. The sequence for Rab13 was N-K-P-P-S-T-D-L-K-T-C-D-K-N-T. The peptide sequence for Rab8 was S-N-Q-G-V-K-I-T-P-D-Q-Q-K-R-S-S-F. In general, the peptide is injected into the popliteal lymph nodes of a rabbit as described (Cooper and Paterson, 2001). Affinity purified antibody (and the corresponding flow-through antiserum) was generated after bleeding of the injected rabbits.

**Western blot Assays**

MDCK and LLC-PK1 cell lysate (1µg/µL) were run on 14% SDS-PAGE gels. Gels were then blotted onto nitrocellulose membranes and decorated with Rab13 flow through antiserum.
(dilutions 1:100, 1:500 and 1:1000) and Rab13 affinity purified Rab13 polyclonal antibody 1:1000. The secondary antibody (at 1:5000) was coupled to horseradish peroxidase and signals were detected by chemiluminescence (Pierce, Rockford, IL).
REFERENCES


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**Education**

Northwestern University- Ph.D., Biochemistry, Molecular Biology, and Cell Biology  
The University of Arizona- M.S., Soil, Water, and Environmental Biology (1998)  
St. Edward’s University- B.S., Biology (1996)

**Research Experience**

Interdepartmental Biological Sciences Program Graduate Student  
Heike Fölsch, Ph.D., Northwestern University  
My current research establishes a role for the small GTPase Rab13 in regulating membrane transport from the trans-Golgi network to recycling endosomes.  
- Microinjection of cDNAs followed by immunofluorescence labeling and confocal microscopy to visualize protein trafficking in epithelial cells  
- Production and use of adenoviruses to express a specific protein in mammalian cells  
- Tissue culture

Soil, Water, and Environmental Science Program Graduate Student  
Charles Gerba, Ph.D., The University of Arizona  
My thesis research focuses on evaluating the ability of three small-scale, on-site subsurface wetlands with different vegetation densities to purify wastewater. Water samples were collected and tested for removal of total coliforms, fecal coliforms, coliphage, *Giardia* and *Cryptosporidium*.  
- Parasite analysis in environmental water samples by immunofluorescence antibody staining and UV/DIC microscopy  
- Point-of-Use device evaluation for parasite, virus, and bacteria removal  
- Culturable techniques with selective and differential media  
- Supervision and training of graduate and undergraduate students

Lower Colorado River Authority, Austin, TX  
1995 to 1996  
Undergraduate Senior Seminar Research Project  
- Research common algae occurrence in polluted streams  
- Identify pollution indicator algae through microscopic analysis

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**Professional Experience**

Environmental Resources Management, Houston, TX  
1998 to 2001  
Environmental Consultant  
- State Superfund Site Project Management  
- Database creation and maintenance  
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**Publications and Presentations**


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Association of Women in Science (AWIS) Chicago Chapter member
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American Association for the Advancement of Science (AAAS) member
BioProfessionals Committee member 2007-2008
Bio Opportunities Committee Chair 2005-2007
IBiS Student Organization, Social Chair 2003-2004